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**PERIODONTAL MEDICINE:
ORAL INFLAMMATORY CONDITIONS
WITH SPECIAL EMPHASIS ON
IMMUNOLOGICAL ASPECTS**

Jeneen Panezai



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PERIODONTAL MEDICINE: ORAL INFLAMMATORY CONDITIONS WITH SPECIAL EMPHASIS ON IMMUNOLOGICAL ASPECTS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Jeneen Panezai

Principal Supervisor:

Docent Per-Erik Engström
Karolinska Institutet, Sweden
Department of Dental Medicine
Division of Oral Diseases
Section of Periodontology

Co-supervisor(s):

Professor Anders Larsson
Uppsala University, Sweden
Department of Medical Sciences
Division of Clinical Chemistry

Professor Karl-Gösta Sundqvist
Karolinska Institutet, Sweden
Department of Laboratory Medicine
Division of Clinical Immunology

Professor Björn Klinge
Malmö University, Sweden
Faculty of Odontology,
Department of Periodontology
and
Karolinska Institutet, Sweden
Department of Dental Medicine
Division of Oral Diseases
Section of Periodontology

Professor Dr. Mohammad Altamash
Altamash Institute of Dental Medicine, Pakistan
Department of Periodontology

Mentor:

Professor Malin Ernberg
Karolinska Institutet, Sweden
Department of Dental Medicine
Section for Orofacial Pain and Jaw Function

Opponent:

Professor Thomas Van Dyke
Harvard School of Dental
Medicine, Boston, USA
Department of Oral Medicine,
Infection, and Immunity

Examination Board:

Professor Lennart Emtestam
Karolinska Institutet
Department of Medicine

Docent Per Ramberg
Gothenburg University
Department of Periodontology

Docent Eva Baecklund
Uppsala University
Department of Rheumatology

ABSTRACT

Systemic effects of periodontal disease have been a subject of interest for the past century, with intense focus converging since the past decade. Both rheumatoid arthritis (RA) and periodontal disease (PD) are immuno-inflammatory diseases with osteolysis as its hallmark feature. Activated T cells are known to modulate osteoclastogenesis. This thesis aimed to analyze the influence of PD on systemic inflammatory and immunological markers in both PD and RA subjects. Periodontal parameters, clinical (PI, BOP, PPD 3–<5mm and PPD ≥5mm) and radiographic (marginal bone loss or MBL) were investigated in four groups: RA with PD, RA without PD, PD and healthy groups. Immunosuppression of T cell activation via targeted surface protein was also studied.

AIMS

Study I aimed to investigate the expression and functional importance of low-density lipoprotein receptor-related protein1 (LRP1) in T cells.

Study II aimed to investigate serum cytokines, chemokines, growth factors, enzymes and costimulatory proteins in association with periodontal conditions in PD and RA subjects.

Study III aimed to investigate the serum markers osteopontin (OPN), tumor necrosis factor receptors 1 (TNFR1) and 2 (TNFR2) receptor activator of nuclear factor-kappa B ligand (RANKL) and RANKL/ osteoprotegerin (OPG) ratio and compare them in PD and RA groups.

Study IV aimed to investigate the severity of both PD and RA and investigate a correlation between glycemia and periodontal disease parameters using ΣPPD Total and ΣPPD Disease index.

RESULTS

Study I showed that T cells shed LRP1, which probably explains the low LRP1 expression in T cells. Shedding of LRP1 antagonizes T cell adhesion to integrin ligands and TCR-induced activation. Integrin ligands and CXCL12 antagonize shedding through a TSP-1-dependent pathway, whereas ligation of CD28 antagonizes shedding independent of TSP-1. The disappearance of LRP1 from the cell surface may provide basic immunosuppression at the T-cell level.

Study II showed significant positive correlations for ST1A1, FGF-19 and NT-3 whereas EN-RAGE, DNER, CX3CL1 and TWEAK associated inversely with BOP, PPD ≥ 5mm and MBL but positively with number of teeth. CD markers (CD244, CD40, CDCP1, LIF-R, IL-10RA, CD5 and CD6) were found to be associated with BOP, shallow and deep pockets, MBL and number of teeth, either directly or inversely. CCL8, CX3CL1, CXCL10, CXCL11, CCL11, CCL4, CCL20, CXCL5, CXCL6, and CCL23 were positively associated with number of teeth. Other growth factors were directly associated with MBL (HGF) and number of teeth (VEGF-A, LAP TGFβ-1).

Study III showed OPN, TNFR1, TNFR2 and RANKL serum levels were the highest in the RA group with PD, while the RA group without PD were comparable to PD subjects only. The RANKL/OPG ratios were comparable between PD group and both RA groups with and without PD. Serum RANKL levels were associated with and PPD ≥ 5mm.

Study IV showed that the indices correlated strongly with number of deep pockets. DAS28 score correlated positively with RF in RA subjects with and without PD. Serum levels of HbA1c were higher in PD, RA with PD and without PD subjects as compared to the healthy group. HbA1c levels associated positively with PPD Total, PPD Disease, and MBL. Tooth adjusted PPD Total correlated with all periodontal parameters except for shallow pockets.

CONCLUSIONS

This thesis shows that periodontal disease is mirrored by a range of systemic immune markers, particularly those involved in inflammation. Furthermore, peripheral osteoclastogenesis is a feature of PD, comparable to RA. Overall, this thesis signifies the peripheral involvement of host immune system in combating PD essentially as an osteolytic disease and the need to approach PD measurement via a novel continuous index. The thesis also shows evidence that LRP1 controls motility, adhesion and activation in T cells.

Key words: periodontal disease, rheumatoid arthritis, inflammation, PPD, MBL, bone loss, cytokines, chemokines, T cell, RANKL, TNF receptors, index, HbA1c

LIST OF SCIENTIFIC PAPERS

- I. **J. Panezai**, E. Bergdahl, K-G. Sundqvist.
T-cell regulation through a basic suppressive mechanism targeting low-density lipoprotein receptor-related protein 1.
Immunology. 2017;152: 308–327.
- II. **J. Panezai**, A. Ghaffar , M. Altamash, K-G .Sundqvist, P-E. Engström, A. Larsson.
Correlation of serum cytokines, chemokines, growth factors and enzymes with periodontal disease parameters.
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- III. **J. Panezai**, A. Ghaffar, M. Altamash, P-E. Engström, A. Larsson.
Periodontal Disease influences osteoclastogenic bone markers in subjects with and without rheumatoid arthritis.
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- IV. **J. Panezai**, A. Ali, A. Ghaffar, D. Benchimol, M. Altamash, B. Klinge, A. Larsson, P-E. Engström.
Probing Pocket Depth Total Index, Glycated Hemoglobin and Disease Severity Measurements in Subjects with and without Rheumatoid Arthritis.
Submitted.

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LIST OF ABBREVIATIONS

Th1	Type 1 T helper cells
Th2	Type 2 T helper cells
Th17	Type 17 T helper cells
Treg	Regulatory T cells
CD	Cluster of differentiation
IFN	Interferon
TNF- α	Tumor necrosis factor alpha
IL	Interleukin
GM-CSF	Granulocyte macrophage - colony stimulating factor
MHC	Major histocompatibility complex
PD	Periodontal disease
RANKL	Receptor activator of nuclear factor kappa-B ligand
TLR2	Toll-like receptor 2
TCR	T cell receptor
BCR	B cell receptor
GAG	Glycosaminoglycan
DC	Dendritic cell
NK	Natural killer cell
HLA	Human leukocyte antigen
MHC	Major Histocompatibility Complex
T2D	Type 2 diabetes
ACPA	Anti-citrullinated protein antibody
CSF	Colony stimulating factor
M-CSF	Macrophage-colony stimulating factor
FGF	Fibroblast growth factor
HGF	Hepatocyte growth facto
EGF	Epidermal growth factor
PDGF	Platelet derived growth factor
IGF	Insulin-like growth factor
NGF	Nerve growth factor
APC	Antigen presenting cell
EDTA	Ethylenediaminetetraacetic acid
NLRP3	Nucleotide-Binding Oligomerization Domain, Leucine Rich Repeat and Pyrin Domain Containing 3

INTRODUCTION

Inflammation

Inflammation is a special state in terms of tissue response to injury that causes a shift in its metabolic status from an anabolic towards a catabolic one. In the strictest sense, these characteristics differ from that of a tissue degenerative process firstly, by the rate with which catabolism exceeds anabolism and, secondly, the rise in the osmotic pressure causing significant fluid accumulation. Therefore, inflammation is almost a pathological process arising as a direct consequence of a tissue insult or injury. To counterbalance it, an opposing reactive process, the anti-inflammatory response, is characterized by proteolysis inhibition (Hiemstra, 2002).

In a healthy state, after inflammation reaches its maximum capacity, an anti-inflammatory response ensues to restore tissue homeostasis, representing a true defensive response (Table 1). Thus, inflammation ensued by an anti-inflammatory response is vital for complete healing which, if not the case, can develop into chronic inflammation and autoimmune diseases (Stankov, 2012).

Table 1. Contrasting features of inflammation and anti-inflammatory process*

Inflammation	Anti-inflammatory response
Stimulated catabolism	Catabolic inhibition
Hypoxia	NYK**
Hyperosmolarity	NYK
Immediate-transient venular response	NYK
Edema formation	Edema resolution
Necrosis	Apoptotic cell clearance
Lysis of colloids	NYK
Immunostimulation	NYK

*adapted from (Stankov, 2012).

** Not yet known

Acute inflammation

Based on onset and duration, inflammation can be categorized into acute and chronic. *Acute inflammation* is characterized by a rapid onset, short duration, and profound signs and symptoms.

Acute inflammation occurs through several stages that are recognized through cellular changes. Initially, there is an increase in blood flow within and around the vasculature at the site of injury. An increase in vascular permeability allows recruited immune cells extravasate into the injured parenchyma where they migrate to the injured cells using gradients of inflammatory molecules via chemotaxis. Upon reaching the site of injury, immune cells proceed to phagocytose microbes and cellular debris which may be present (Rubin et al., 2009). The ultimate goal of the acute inflammatory response is clearance of damaged host cells and removal of pathogens. However, some recruited immune cells such as macrophages and dendritic cells travel to local lymph nodes where, as APCs, they process and present degraded phagocytosed debris advancing the immune response from that of an innate to an adaptive one.

Some of the key features of acute inflammation, which aim to culminate in the formation of an exudate, are brought about by several bioactive molecules. The most profound effects are on vasculature and leukocyte recruitment (Figure 1).

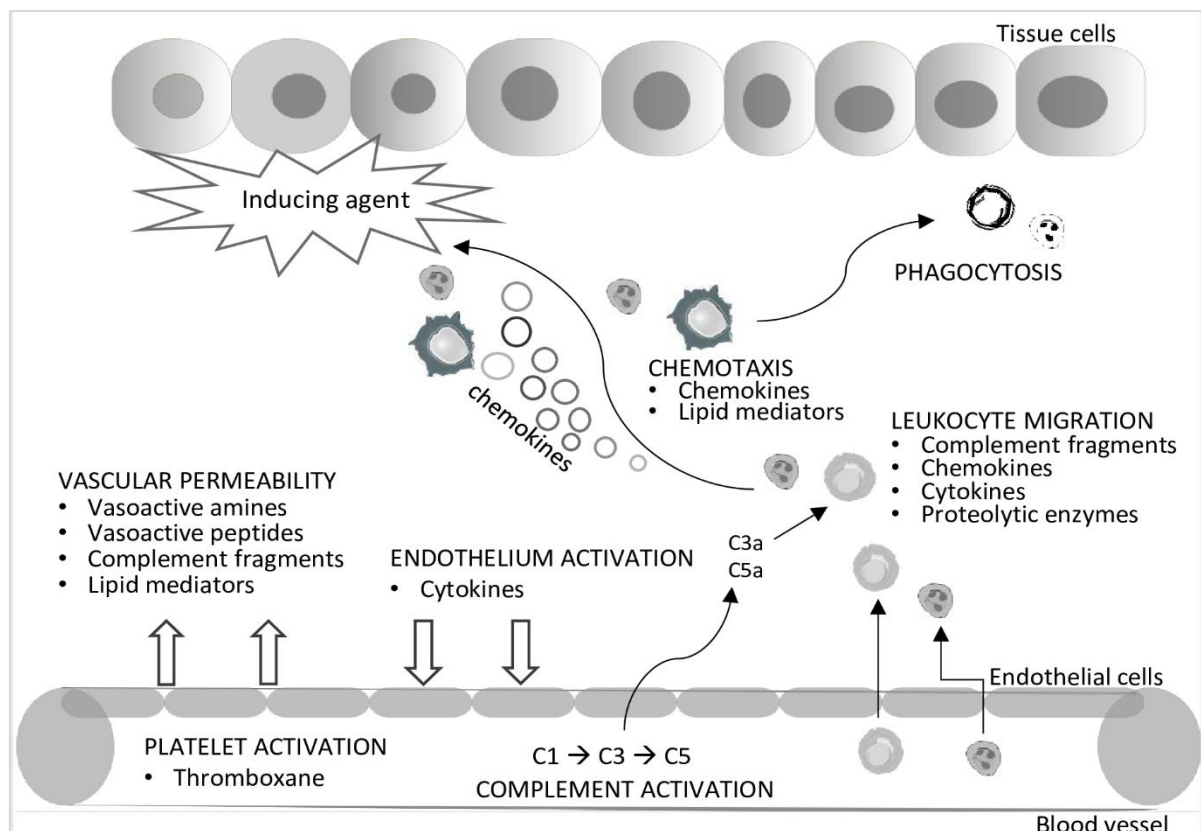


Figure 1. A simplified overview of the inflammatory mediators involved in the key features of acute inflammation (Medzhitov, 2008).

 Tissue macrophages
  Neutrophils
  Leukocytes
  Phagocytosed cells

Chronic inflammation

In contrast, *chronic inflammation* is slow in onset and longer in duration with variable intensity of signs and symptoms. Chronic inflammation can follow acute inflammation, but it can also occur as a symptomless low-grade, prolonged response to an inciting agent.

A transition to a pattern of chronic inflammation develops following weeks of an unresolved inflammatory process. This is associated with a shift in the demographic of immune cells at the site of injury.

The prime feature of chronic inflammation is the prominent presence of monocyte/macrophages, lymphocytes, plasma cells, fibroblasts and vascular endothelial cells at the site of injury (Rubin et al., 2009). For inflammation to be resolved, an active process of resolution must be brought about via the biosynthesis of *pro-resolving* mediators which can modulate key events of inflammation in order to restore tissue homeostasis (Serhan et al., 2008).

Autoimmunity and autoinflammation

Autoimmunity can be described as self-directed injury, whereby B and T cell responses in primary and secondary lymphoid organs lead to breaking of tolerance. This results in a strong immune reactivity towards self-antigens. Organ-specific pathologic autoantibodies may predate clinical disease expression by years before tissue damage is evident. Most autoimmune diseases have an inflammatory component with effector mechanisms that involve CD4 T cell subpopulation (Th1, Th2, Th17, Treg, Th9 and Th22) (Ivanova et al., 2015). According to recent studies, several cytokines (IFN, TNF- α , IL-1 β , IL-10, IL-13, IL-17, GM-CSF) are

involved in the pathogenesis of systemic lupus erythematosus, polymyositis, rheumatoid arthritis and systemic scleroderma (Higgs et al., 2012).

Autoinflammatory diseases are clinically manifested conditions marked by abnormally increased inflammation mediated by cells and molecules of the innate immune system. The dysregulation mainly involves the failure to process and control the secretion of cytokines IL-1 β and IL-18. This is related to mutations in NLRP3, a component of a macromolecular complex known as the inflammasome. The inflammasome mediated activation of caspase-1 is responsible for IL-1 β and IL-18 processing (Hoffman and Broderick, 2016). Recently, some evidence has shown that the same pathogenetic mechanism responsible for the activation of innate immunity in inherited autoinflammatory diseases may also play a key role in sustaining inflammation in several multifactorial illnesses, such as T2D (Ciccarelli et al., 2014). Typically, autoinflammatory diseases have no associations with HLA or MHC class II haplotypes, and there is an absence of autoreactive T cells or autoantibodies (Shaw et al., 2011, Doria et al., 2012).

Periodontal inflammation

Most inflammatory diseases of the oral cavity involve the tissues of periodontium. The teeth emerge into the oral cavity after crossing bone and connective tissue as well as epithelial tissues. In the environment of the oral cavity, these hard structures are surrounded by a biofilm comprising of a vast collection of bacteria known to exist outside the colon. Such an architectural arrangement of soft and hard tissue compartments may or may not act jointly during the inflammatory responses.

The two common diseases affecting the health of the periodontium are gingivitis and PD. Gingivitis, or inflammation of the gingiva, is limited to the soft tissues comprising of epithelial and connective tissue. The inflammatory changes in gingivitis are reversible with adequate hygiene measures. In the case of periodontal disease, the inflammatory process involves the periodontal ligament and alveolar bone. The progression from a reversible gingivitis to an irreversible loss of bone and ligamental tissues around the tooth has been described in terms of histopathological stages (Page and Schroeder, 1976). With the passage of time, the description was modified. The histopathological description is shown in Table 2 (Kinane and Lindhe, 1997).

Periodontal health is likely to be challenged by a dysbiotic oral microbiota but ultimately, periodontal destruction is the result of a dysregulated immune response.

Role of immune cells in periodontal disease

In healthy periodontal tissues, local immunity is orchestrated by the presence of antigen presenting cells (macrophages and dendritic cells), T lymphocytes (mostly CD4-positive helper T lymphocytes), and a comparatively lower number of neutrophils, B-cells and plasma cells. During inflammation, an increase is seen in most of these cells.

Neutrophils comprise of >95% of leucocytes in the gingival crevice. Co-ordinated and regulated recruitment of neutrophils is vital to periodontal health. In patients with PD, neutrophils have shown decreased chemotactic accuracy and propensity towards increased secretion of pro-inflammatory cytokines including membrane bound RANKL, CCL2 and CCL20 which recruit Th17 cells and APRIL, a B-cell stimulator and proliferation-inducing ligand (Chakravarti et al., 2009, Pelletier et al., 2010).

Macrophages are present in low numbers in healthy periodontal tissues, however they have been shown to positively correlate with the extent of collagen breakdown in PD (Younes et al., 2009, Segulier et al., 2001). They have also been detected in deep periodontal pockets and sites associated with disease progression (Allam et al., 2011). Few studies have demonstrated that TLR2 signaling in macrophages is crucial for TNF-alpha-dependent osteoclastogenesis (Ukai et al., 2008). Macrophages have a role in the resolution of inflammation mainly by removing apoptotic neutrophils (efferocytosis) (Ortega-Gomez et al., 2013). This may be a compromised mechanism in PD pathogenesis since PD is a chronic disease in which inflammation is not resolved in a timely manner.

T lymphocytes can be polarized into distinct T-helper cellular subsets upon activation by APCs (Figure 2). The balance between functional T cell subsets is crucial for maintaining homeostasis. Several studies in humans show Th1 cells are associated with stable lesions whereas advanced or progressive lesions are associated with Th2 cells in PD but this dichotomy was too simplistic to clarify PD pathogenesis. Tregs are strong inhibitors of osteoclast formation but under inflammatory conditions, the immuno-suppressive functions of these CD4 Tregs are diminished by the abundance of pro-inflammatory cytokines (Valencia et al., 2006). These CD4 Tregs are present in low numbers in the gingival tissues of subjects with PD where some may switch to Th17 phenotype (Okui et al., 2010). The role of Th9 and Th22 subsets have not been studied as extensively in PD, however IL-22 has been linked to disease severity and bone loss (Díaz-Zúñiga et al., 2017). Recent studies regarding Th cells have revealed that these cells are neither inflexible nor strictly committed but rather exhibiting ‘plasticity’ in their phenotype as dictated by conditions activated by different cytokines (O’Shea and Paul, 2010).

Table 2. Clinical and histopathological stages of periodontal disease

<i>Clinical condition</i>	<i>Time (days)</i>	<i>Histopathological condition</i>
Pristine gingiva	-	Little or no inflammatory infiltrate
Normal healthy gingiva	4 days	Initial lesion: <ul style="list-style-type: none"> • vasculitis below junctional epithelium (JE) • exudation of fluid into connective tissues and gingival sulcus • increased migration of neutrophils and leucocytes • increased perivascular collagen loss
Early gingivitis	7 days	Early lesion <ul style="list-style-type: none"> • accumulation of lymphoid cells below JE • cytopathic changes in resident fibroblasts • further loss of collagen • early proliferation of basal cells of the junctional epithelium
Established gingivitis	21 days	Established lesion <ul style="list-style-type: none"> • increase in plasma cells • presence of immunoglobulins in the connective tissues, JE and gingival crevice • continued loss of collagen fibers and fibrous CT matrix • proliferation, apical migration and lateral extension of the JE
Periodontal disease	-	Advanced lesion <ul style="list-style-type: none"> • persistence of features in established lesion • formation of periodontal pocket • significant bone loss • loss of periodontal ligament

*adapted from Kinane and Lindhe, 1997.

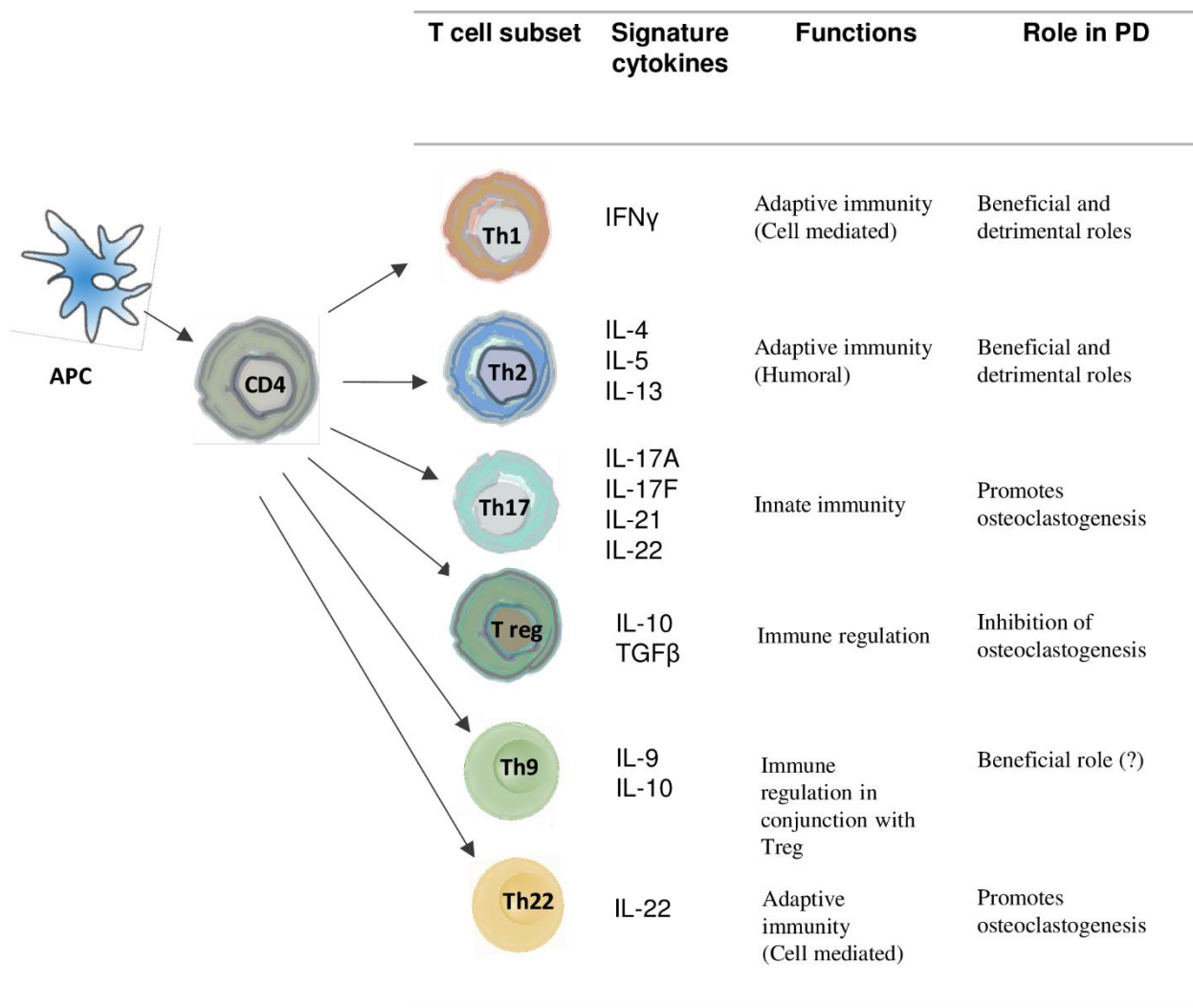


Figure 2. T lymphocyte subsets functions and roles in PD. Differentiation of naïve CD4 T cells into different types of T helper subsets (Th) or inducible regulatory T cells (T regs) and their roles in periodontal disease (PD). APC = antigen presenting cell. (Okui et al., 2014, Díaz-Zúñiga et al., 2017).

B lymphocytes

In established periodontal lesions, there is a clear dominance of B- lineage cells, comprising of up to 60% of the total leukocyte infiltrate (Thorbert-Mros et al., 2015). In severe PD, tissue samples show B lymphocytes predominating over plasma cells and T lymphocytes. A majority of these B lymphocytes were found to express CD5 (Donati et al., 2009). The presence of high numbers of B lymphocytes in PD lesions seems to be linked to polymicrobial antigenic stimulation within the oral biofilm. To contravene this antigenic challenge, local B lymphocytes are likely to be induced and activated via both T dependent and T independent pathways. The role of B cell antibody response in PD is still not clear as studies have shown that subjects with high antibody titers against *P. gingivalis* have more severe alveolar bone loss. This may be attributed to the antibody response in PD being of low affinity (Whitney et al. 1992, Kinane et al.. 1999).

Osteoclasts

Osteoclasts (OCs) can be described as specialized polykaryons that belong to the macrophage lineage (Boyle et al, 2003). Both mature OCs and osteoclast precursors respond to inflammatory cytokines as part of regulation by the immune system. They are, therefore, immune cells. It has been shown that in common with other cells of the immune system, osteoclasts also share a requirement for costimulatory signals that are mediated by immuno-receptor tyrosine-based activation motifs (ITAMs) (Koga et al., 2004). In osteoclasts, this co-stimulation is needed for RANKL-induced differentiation followed by bone resorption (Koga et al., 2004). Like innate immune cells, OCs express the inflammasome complex as well. OCs are formed from the

fusion of from peripheral blood-borne mononuclear cells that arise from precursor cells in the bone marrow. These cells are the same mononuclear precursor cells as macrophages and dendritic cells (DCs), therefore it has been demonstrated in vitro that OCs can act as functional APCs as they expressed MHC molecules (HLA-ABC and HLA-DR), costimulatory molecules (CD80, CD86, and CD40) and that the expression of these molecules could be up-regulated by IFN- γ . They also play a role in bone formation by releasing cellular products, termed as 'clastokines', which are capable of stimulating osteoblast bone formation. OCs have a vital role in the pathogenesis of PD since bone loss is a hallmark of this disease. The OC precursors circulate in blood, extravasate and settle in the bone, promptly differentiating into OCs in the presence of M-CSF and RANKL (Muto et al., 2011). A recent study showed via nucleoside labelling of precursor cell nuclei that > 90% of OCs found in ligation induced PD were derived from OC precursors formed before the induction of PD (Lee et al., 2015).

Cytokines, chemokines and growth factors

Inflammation is controlled by a host of extracellular mediators and regulators which include cytokines, chemokines and growth factors.

Cytokines are cellular messengers that are highly active within the environment of their cellular source(s). Structurally, they are synthesized as either proteins, glycoproteins or polypeptides. Majority of them have immunologically modifying effects. They are mostly secreted but may also be expressed on the cell membrane. There are no structural differences between cytokines bound to cell membranes and those that are secreted. The membrane-bound cytokines can be cleaved at the extracellular part by metalloproteases and, therefore, secreted. However, their secretion is not continuous in nature as they are mostly produced in response to cell activation. The efficacy of cytokines as cellular messengers is established through an interaction with their receptors which results in a signal transduction that results in a fast or a slow action on the target cell. **Chemokines** are an excellent example of fast action inducing signal transduction. They are cytokines which are involved in chemotaxis, a process of attracting cells to a specific site via establishment of a chemically stimulating gradient. This is a vital process in an immuno-inflammatory response for controlling leukocyte trafficking. Most chemokines bind to several receptors and each chemokine receptor can bind more than one ligand. Based on their primary structure, there are four families of receptors (R): XCR, CCR, CXCR, and CX3CR (X stands for any amino acid; C denotes cysteine). The chemokine ligands (L) are separated into four groups: XCL, CCL, CXCL, and CX3CL (Dembic, 2015).

Cytokines which influence growth and tissue regeneration can be classified according to the extent of their influence such as those on:

1. Single line of cells: CSF, G-CSF, M-CSF, GM-CSF.
2. Specific tissues: FGF, HGF, EGF, PDGF, IGF, NGF.
3. All tissues: TNF- α , TNF- β and TGF- β (Dembic, 2015).

Cytokines play an important part in the host response to PD as they have putative roles in terms of tissue destruction or protection (Figure 3).














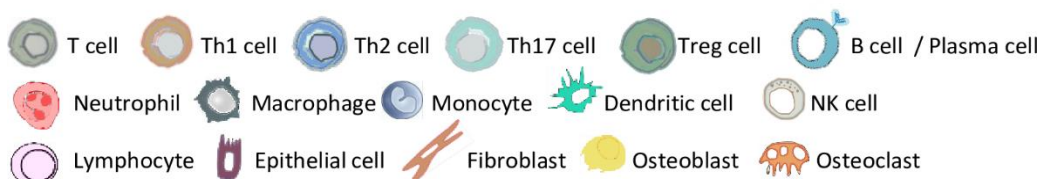
Cytokine / chemokine	Cellular source	Function	Response in periodontal inflammation
IL- β		Pro-inflammatory and hematopoietic effects. Promotes development of the Th17 phenotype of helper T cells	Increased; induces bone resorption
IL-4		Growth and development of B lymphocytes. Promotes development of Th2 type of immune responses regulating humoral immunity	Low to high
IL-6		Pro-inflammatory, produces acute phase proteins in liver cells, helps formation of the Th17 phenotype of helper T cells from the Th0 stage	Increased; osteoclastogenic
IL-10		Inhibits the development of Th1, boosts the survival and proliferation of B cells and production of antibodies	Increased
IL-12		Key factor in the development of Th1 and inhibition of Th2 T helper cells, enhances the defensive action of T and B cells	Low to high
IL-17		Acts synergistically with other pro-inflammatory signals via TNF- α for induction of GM-CSF, and with CD40-ligand for secretion of IL-6, IL-8, CCL5 and CCL2	Low to high; osteoclastogenic
TNF- α		Pro-inflammatory, upregulates adhesion molecules, chemokine production, regulation of IL-1 β and IL-6, increases MMP and RANKL expression	Increased; osteoclastogenic
TGF- β		Immunosuppressive leading to reduction in proliferation of B, T, and NK cells, as well as their function; antagonizing effects of pro-inflammatory (TNF-like) cytokines	Low to high
IFN- γ		Promotes Th1 development, inhibits Th2 development, stimulates development of B lymphocytes, major activation factor of macrophages, suppresses development of Th17 cells	Low to high
CXCL8		Attracts and activates neutrophils, promotes angiogenesis	Increased
CCL2		Attracts monocytes and activates them. Also attracts basophils, activated T cells, NK cells, and immature dendritic cells	Increased
RANKL		Binds with RANK to induce signaling for regulation of osteoclast formation, activation and survival in normal bone modeling and remodeling	Increased
OPG		Inhibitor of RANKL	Decreased

Figure 3. Cytokines and their documented response in periodontal inflammation (Garlet et al., 2012).



T lymphocyte regulation

An effective T cell immune response requires an antigen-specific signal through the TCR along with simultaneous antigen non-specific signaling via a co-stimulatory receptor. T cells migrate to the periphery and enter the naive T cell pool after undergoing a selection check in the thymus. In the periphery, naive T cells are continuously recirculating between secondary lymphoid organs and blood through the lymphatic system. In terms of function, these cells have yet to encounter their associated antigen which, upon doing so, will cause them to differentiate and proliferate into effector or memory T cells. Effector T lymphocyte motility, adhesion, and activation depend on certain factors which would prevent it from being activated under transient adhesive interactions (van den Broek et al., 2018, Lui Z et al., 2009).

T regs are specialized cells for suppressing immune responses towards self and non-self-antigens. Mature T regs can be induced in the periphery but the majority of circulating Tregs are generated in the thymus. T regs express Foxp3 which is a transcription factor required for the differentiation of a TCR positive T cells into T reg cells. T reg mediated suppression comprises of secretion of immunosuppressive cytokines, and modification or killing of APC. Although self-reactive T cells are deleted in the thymus and the periphery, sometimes elimination is not carried out completely. This then requires immune suppression mechanisms to keep auto reactive T cells in check. Specialized cells and/ or cytokines are major elements of this actively carried out immune suppression. Also known as inhibitory immune receptors, immune checkpoints serve as negative regulators of the immune response, thereby protecting the host from excessive inflammation. Cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and programmed cell death protein1 (PD1) are known immune checkpoints. CTLA-4 competes with co-stimulatory molecule CD28 for co-stimulatory ligands and attenuates the early activation of naive T cells. PD1 directly interferes with TCR signaling and can inhibit T cells at the effector stage. The expression of PD1 has been associated with T cell exhaustion and has been shown to be expressed by gingival CD8 T cells (Figueira et al., 2009). CTLA-4 being a crucial Treg related molecule, has been shown to be expressed by only a small number of cells in periodontal lesions (Orima et al., 1999).

The importance of transforming growth factor-beta (TGF- β) in T cell regulation is immense due to its ability to stimulate naive T cells to differentiate into Tregs. The differentiation takes place via the induction of Foxp3 expression allowing them to acquire regulatory roles which comprise of suppressing effector T cell proliferation and function. TGF- β is a pleiotropic cytokine known to suppress genes involved in T-cell differentiation and function. It can also suppress the production of pro-inflammatory cytokines and stimulate T-cell production of IL-10 (Kitani et al., 2003, Johnston et al., 2016).

EPIDEMIOLOGY OF PERIODONTAL DISEASE

Prevalence of PD

Severe PD was reported to have an age-standardized global prevalence of 7.4% in 2015 (Kassebaum et al., 2017). In an earlier report on the global burden of diseases, severe PD was the sixth most prevalent disease amongst 291 diseases (Murray et al., 2012). The global burden of PD has increased by 74.9% from 1990 to 2015 becoming the leading cause of tooth loss in adults (Kassebaum et al., 2017). As a result, it contributes to edentulism and masticatory dysfunction (Petersen and Ogawa, 2012). Southeast Asia is also one of the regions which has severe PD as the leading cause of disability adjusted life-years (DALYs) (Marcenes et al., 2013). Some studies have reported a prevalence ranging from 60 to 100 % (Shaju et al, 2011). According to the WHO oral health situation analysis of Pakistan, an astounding 93% of individuals above the age of 65 have periodontal disease (Khan et al., 2004).

Risk assessment for PD

In order to assess the likelihood for an individual to develop PD, factors, determinants and predictors are important in identifying ‘potential risk’. A risk factor is an environmental exposure, an aspect of personal behavior or inherited characteristics which are known to be associated with a health related condition. Risk factors can be classified as modifiable or non-modifiable. Modifiable risk factors are environmental or behavioral in nature whereas non-modifiable risk factors are intrinsic to the individual and therefore not changeable. Risk determinants are non-modifiable risk factors. Risk predictors are clinical or biological markers indicative of disease progression with no etiological relationship to the disease (Figure 4) (Last et al., 2001, Van Dyke and Dave, 2005). Table 3 shows the literary evidence for factors, determinants and predictors of risk associated with PD development.

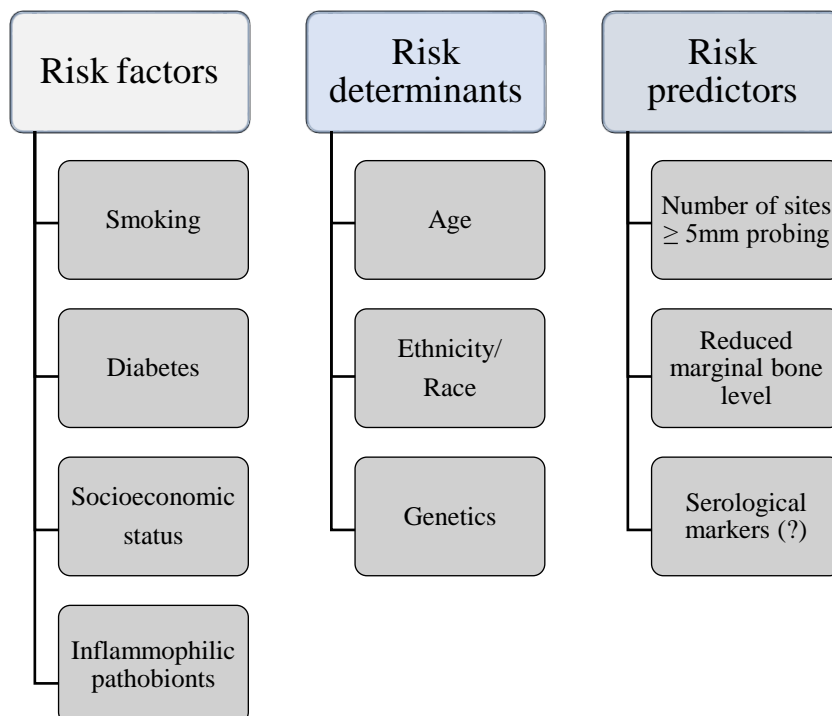


Figure 4. Risk factors, determinants and predictors implicated in periodontal disease development.

Table 3. Evidence for the role of risk factors, determinants and predictors in periodontal disease

Smoking	<ul style="list-style-type: none">• Associated with increased alveolar bone loss, development of periodontal pockets and tooth loss (Bergström et al., 1994).• Cumulative effect of longer duration of smoking resulting in greater periodontal destruction (Grossi et al., 1995).• Periodontal therapy in smokers is associated with less reduction in probing depth and smaller gains of clinical attachment than in nonsmokers (Patel et al., 2012, Jin et al., 2000).
Diabetes	<ul style="list-style-type: none">• Higher prevalence and severity of periodontal disease in patients with type 1 and type 2 diabetes (Hodge et al., 2012, Taylor et al., 2010).• Relative risk of incidence of periodontal disease is 2.6 (95% confidence interval: 1.0–6.6) in patients with type 2 diabetes compared with people without diabetes (Nelson et al., 1990).• Severity of periodontal conditions is increased in poorly controlled diabetics as compared to pre-diabetics (Altamash et al., 2013).• Treatment of periodontal disease results in consistent reduction in glycated hemoglobin (Altamash et al., 2016, Simpson et al., 2010).
Socioeconomic status	<ul style="list-style-type: none">• Social hierarchies create levels of psycho-social stress which are manifest in health gradients (Wilkinson et al., 1999).• SES variables account for ~50% of the differences in PD prevalence at 35–44 years of age (Hobdell et al., 2003).• Distinct pattern of health-related behavior, knowledge and health attitudes reflect differences in periodontal health (Peterson, 1990).
Inflammophilic pathobionts	<ul style="list-style-type: none">• Alteration of the periodontal microbiota from a symbiotic to a dysbiotic state in a susceptible host (Hajishengalis, 2014a).• Presence of keystone pathogens such as <i>P. gingivalis</i> can shift the host- microbe homeostatic balance toward dysbiosis (Hajishengalis et al., 2011).• Host tissue inflammation allows growth of ‘inflammophilic’ bacteria that thrive and contribute to further pathogenesis (Abusleme et al., 2013, Hajishengalis, 2014b).
Age	<ul style="list-style-type: none">• Increasing age associated with an increase in the prevalence, extent and severity of periodontal attachment loss (Eke et al., 2012, Machtei et al., 1999).• The rate of radiographic alveolar bone loss increases in the third to fifth decades (Grossi et al., 1995).
Ethnicity and sex	<ul style="list-style-type: none">• Ethnicity seems to be associated with periodontal disease, possibly through socioeconomic linkage (Borell & Crawford, 2012).• Greater risk for destructive periodontal disease in men than women (Shiau & Reynolds, 2010).
Genetics	<ul style="list-style-type: none">• Familial aggregation studies reveal that PD in young individuals has a stronger genetic link (Meng et al., 2011).• Polymorphisms in the interleukin-1, interleukin-6, interleukin-10 and CD14 genes are restricted to certain populations (Laine et al., 2010).• Epigenetics play a role in PD (Martins et al., 2015).
Pocket depth	<ul style="list-style-type: none">• Number of sites with deep pockets (≥ 5mm) are risk predictors for progression of PD (Van der Velden et al., 2006).
Marginal bone loss	<ul style="list-style-type: none">• Reduced marginal bone level in younger individuals represents higher risk of further bone loss (Bahrami et al., 2007).
Serological markers	<ul style="list-style-type: none">• Presence of periodontal disease related to high systemic inflammatory protein levels (Dye et al., 2005).• Serum CRP, IL-6, Oncostatin M, MMP-8 and MMP-9 have been identified as biomarkers with great potential for monitoring response to periodontal disease progress (Stathopoulou et al., 2015).

Periodontal Parameters

PD parameters comprise of bleeding on probing, probing pocket depth and radiographic findings (American Academy of Periodontology Task Force Report. 2015).

Plaque/ Oral biofilm

The microbial biofilm has been extensively studied, identifying up to 800 different species in human dental plaque so far (Lourenco et al., 2014). The presence of plaque can induce inflammatory changes in the gingiva leading to noticeable symptoms that include gingival swelling, redness and bleeding with tooth brushing and chewing. Its presence in abundance is an important parameter to identify in terms of therapeutic intervention (Murakami et al., 2018).

Bleeding on probing

In order to assess the state of gingival tissues, BOP is used as a clinical parameter. BOP is measured as bleeding provoked by inserting a probe to the bottom of a pocket. The absence of BOP is a clinical indicator of periodontal health or stability following periodontal therapy (Lang and Bartold, 2018).

Periodontal Pocket depth

The space found between a pathologically deepened gingival sulcus around a tooth is known as a periodontal pocket (Bosshardt, 2018). Increased depth of the periodontal pocket is a clinical finding that reflects breakdown of periodontal fibers, loss of cementum and bone. It is important to describe the biological significance of periodontal probing. Under healthy conditions, the lateral wall of the gingival crevice is lined by the sulcular epithelium in the coronal part and the junctional epithelium in the apical part and measures 0.5mm (Bosshardt, 2018). The junctional epithelium lines the bottom of the sulcus in the region of the cemento-enamel junction (Mombelli, 2005). Probing is performed to delineate the depth of the pocket, measured in millimeters, by passing a narrow-diameter probe gently, but firmly, between the tooth and gingiva.

Radiography

Radiographs provide information regarding bone level and bone loss that cannot be gained through clinical examination. The position of the alveolar bone crest is imaged for estimation of the degree of bone loss. Information regarding the pattern of bone loss, either vertical or horizontal, can also be gained from radiographs (Hirschmann, 1989). The normal position of alveolar bone crest lies within 2 mm from the cemento–enamel junction as a diagnostic threshold (Hansen et al., 1984, Hausmann et al., 1991).

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease influenced by both genetic and environmental factors (Angelotti et al., 2017). In confirmed cases of RA, the course of disease can vary from slowly progressing and overall mild disease, remaining a predominantly local condition to aggressively progressive with multiple joint involvement and erosion with systemic involvement (Forslind et al., 2004). There is no known etiological factor for RA, however genetic susceptibility plays a significant role (Malmström et al., 2017).

EPIDEMIOLOGY

Most epidemiological studies in RA have been done in Western countries, showing a prevalence of RA in the range of 0.5–1.1% (Tobón et al., 2010). In the city of Karachi, Pakistan, the prevalence of RA is reported to be 0.14% as compared to that of northern Pakistan where the estimated prevalence is 0.55% (Hameed et al., 1997).

Risk factors

Genetics: RA has a strong genetic component where the phenotypic variance due to genetic variance is ~60% (MacGregor et al., 2000). This is mostly seen in RA patients who are positive for anti-CCP antibodies, whereas estimates in seronegative disease are lower (Padyukov et al., 2011, Viatte et al., 2016). Specific genetic loci show a very strong association with RA (Gregersen et al., 1987). These are MHC (also known as HLA) molecules that may contain the shared epitope which is a specific amino acid motif commonly encoded by alleles of the HLA-antigen D related (DR) locus. Alleles known to be associated with the risk of developing RA are HLA-DRB1*01 and HLA-DRB1*04 (Weyand et al., 1992).

Epigenetics: The genetic risk associated with HLA variants is partly due to epigenetic changes that entail altered DNA methylation (Liu et al., 2013). Environmental factors can induce changes in cellular function through DNA methylation.

Smoking: The association between tobacco and RA is strongest in anti-CCP positive individuals with at least one copy of the shared epitope. Interaction between the shared epitope and smoking causes a 20 fold greater risk of developing RA as compared to non-smokers (Källberg et al., 2011). Smoking status is also an important factor as current smokers have increased levels of pro-inflammatory cytokines and higher disease activity (Sokolove et al., 2016).

Ethnicity and sex: RA clinical manifestations tend to vary between ethnic groups. This may be explained by ethnic variation in terms of the frequency and types of HLA-DRB1 alleles containing the SE (del Rincón et al., 2003). In terms of gender predilection, the cumulative risk of developing RA is twice as high for women as compared to men (3.6% for women versus 1.7% for men) (Crowson et al., 2011). The stimulatory and regulatory effects of estrogen in localization of Th17 cells during the development of arthritis on the immune system might account for sex as a risk factor (Andersson et al., 2015).

Microbiota: *Porphyromonas gingivalis* is a resident microbe in the subgingival sulcus of the oral cavity. It requires energy from the fermentation of amino acids (Bostanci and Belibasakis, 2012). Research focused on this opportunistic pathogen has postulated an etiological role in the development of RA, specifically in anti-citrullinated protein/peptide antibody positive patients (Rosenstein et al., 2004). This is based on its unique property to express a citrullinating peptidylarginine deiminase (PAD) enzyme. The PAD enzyme is capable of converting arginine residues in proteins to citrulline which consequently alters protein structure and function. Aside from periodontal microbiota, the gut microbiota in new-onset, DMARD naive RA patients had a significantly higher abundance of *Prevotella copri* (Scher et al., 2013). In a recent study, peptides of two novel autoantigens were isolated from HLA-DR molecules of patients with RA that shared the sequence homology with peptides of *Prevotella* and other gut bacterial species (Pianta et al., 2017). Apart from bacteria, the

Epstein–Barr virus (EBV) infection has also been associated with RA as there are reports demonstrating that EBV load is almost 10-fold higher in RA patients than in healthy controls (Balandraud et al., 2003).

Clinical diagnosis

Early RA is characterized by synovial inflammation based on mononuclear cell infiltration, with increased CD4 positive T cells and macrophage activation. The autoimmune response triggers synovial inflammation inducing cytokine production and increased synovial vascularity perpetuating the disease and ultimately causing joint destruction (Figure 5).

Diagnosing RA is an individualized process solely based on the interpretation of the rheumatologist. In the absence of standardized diagnostic criteria, classification criteria that include clinical manifestations and serological assays (autoantibody and acute-phase protein levels) help inform clinical diagnosis.

Serology

Autoantibodies to IgG (rheumatoid factor or RF) and cyclic citrullinated proteins (anti-CCP) are an important characteristic of RA (seropositive RA), but not for all individuals, as some are negative for these autoantibodies (seronegative RA). This may be seen as a major drawback for these two biomarkers since they cannot be detected in seronegative RA cases. Compared with anti-CCP, RF presents higher sensitivity for established disease, with a relatively low specificity. The combination of RF and anti-CCP assays approach a positive predictive value of ~100 %, which is much higher than the value of either of the tests alone (Taylor et al., 2011). The presence of both RF and anti-CCP have been associated with progressive disease (Syversen et al., 2008).

Joint involvement

The joints involved in RA include the metacarpophalangeal joints, proximal interphalangeal joint of the hands and feet, wrist, ankle, elbow, shoulder, knee and hip joints (Smolen et al., 1995). The high inflammatory nature of RA causes articular cartilage and periarticular bone degradation.

Systemic and extra articular manifestations

RA is associated with an increased acute-phase response which can lead to a number of extra-articular manifestations over a period of time. Second to cardiovascular disease, interstitial lung disease is one of the most severe extra-articular manifestations of RA. RA may additionally be accompanied by secondary Sjögren syndrome, secondary amyloidosis or lymphoma (Smolen et al., 2018).













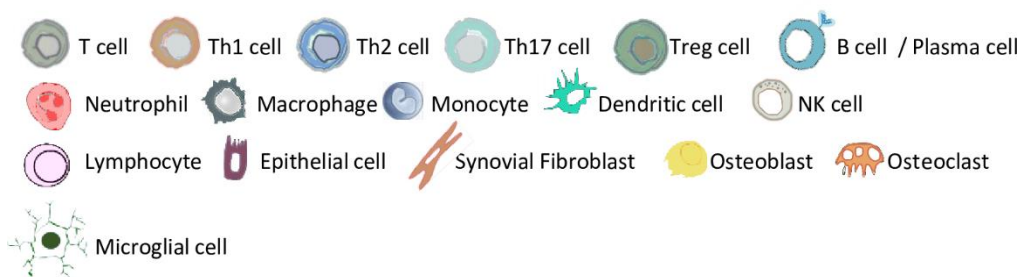
Cytokine / chemokine	Cellular source	Role in rheumatoid arthritis
IL- β		Osteoclast activation, induces bone resorption, enhanced endothelial-cell adhesion molecule expression
IL-6		B cell proliferation and antibody production, T cell proliferation and cytotoxicity, osteoclastogenic, increased hepatic acute phase response
IL-10		Decreased T cell cytokine release, MHC expression and DC activation; increased Treg cell maturation
IL-12		Key factor in the development of Th1; T cell and NK cell cytotoxicity; B cell activation
IL-17		Osteoclastogenic, increased fibroblast and leucocyte cytokine production, decreased cytokine GAG synthesis
TNF- α		Osteoclastogenic, increased monocyte activation, T cell clonal regulation and TCR dysfunction and cytokine release
TGF- β		Matrix maintenance and fibrosis, Treg proliferation, early macrophage activation then suppression, early phase leucocyte chemoattractant
IFN- γ		Increased macrophage and lymphocyte activation, differentiation and survival; broad range of immunomodulatory effects
CXCL8		Key mediator of ACPA-induced OC activation, angiogenesis, neutrophil recruitment
CCL2		TNF-mediated osteoclast differentiation of peripheral blood monocytes, leukocyte migration into the synovium, endothelial cell chemotaxis and angiogenesis
RANKL		Osteoclast maturation and activation, bone resorption; modulates T cell-dendritic cell interactions
OPG		Decreased or low expression allowing RANKL-induced bone loss

Figure 5. Cytokines and their role in rheumatoid arthritis (McInnes and Schett, 2007).



CLASSIFICATION CRITERIA

Classification criteria are standardized definitions that are intended to identify the majority of patients with key features of the condition shared amongst them for research purpose. The goal of classification criteria therefore differs from that of diagnostic criteria (Aggarwal et al. 2015). The current classification criteria are those by the American College of Rheumatology (ACR) and the European League against Rheumatism (EULAR) established in 2010. The ACR/EULAR 2010 criteria have been developed for a population of individuals presenting with at least one clinically swollen joint which cannot be explained by any other disease (Aletaha et al, 2010).

AIMS OF THESIS

GENERAL AIM

The general aim of this thesis was to investigate the systemic inflammatory burden in subjects with periodontal disease, with special emphasis on host immuno-inflammatory responses in terms of inflammatory, immunological and exploratory markers.

SPECIFIC AIMS

Study I

To show that T cell motility, adhesion, and activation depend on the large transmembrane cell surface receptor low-density lipoprotein receptor-related protein 1 (LRP1) and its ligand thrombospondin-1 (TSP-1).

Study II

To investigate serum cytokines, chemokines, growth factors, enzymes and costimulatory proteins in association with periodontal conditions in PD and RA subjects.

Study III

To investigate the serum markers receptor activator of nuclear factor-kappa B ligand (RANKL), osteopontin (OPN), tumor necrosis factor receptors 1 (TNFR1) and 2 (TNFR2), osteoprotegerin (OPG) and RANKL/ OPG ratio and compare them in PD and RA groups.

Study IV

The aim of this study was to investigate the severity of both PD and RA and investigate correlation between glycemia and periodontal disease parameters using a PPD Total index.

ETHICAL CONSIDERATIONS

The research project was approved by the ethics committee of the Altamash institute of Dental Medicine, Karachi, Pakistan and the Regional Ethical Review Board in Stockholm, Sweden. It was conducted in accordance with the Declaration of Helsinki. An informed written consent was obtained in the native language Urdu and English as per requirement, from all participants.

MATERIAL AND METHODS

Patients

In *study II* and *III*, 38 patients suffering from RA were included (mean age \pm SD: 46.1 ± 11.9 years). In *study IV*, a total of 47 RA patients were included (mean age \pm SD: 46.1 ± 11.2 years) as shown in Table 4. They were referred from the Department of Rheumatology at Habib Medical Centre, Karachi, Pakistan.

The number of subjects with PD were 38 in *study II* and *III* (mean age \pm SD: 47.4 ± 9.3 years). In *study IV*, a total of 51 subjects were included (mean age \pm SD: 47.9 ± 9.5 years) (Table 4). Inclusion criteria: 18 years and above in age. Exclusion criteria: individuals suffering from osteoarthritis, gout, and/ or treatment with antibiotics in the last three months and / or undergoing treatment for periodontal disease during the last six months were excluded. Edentulous persons were excluded.

Healthy individuals

Healthy individuals attending the Altamash Institute of Dental Medicine were selected as controls. In *study II* and *III*, 14 healthy controls were included (nine males and five females, range: 35 to 60 years; mean age \pm SD: 44.4 ± 6.6 years). For *study IV*, a total of 20 controls were included (12 males and eight females, mean age \pm SD: 43 ± 6.3 years). The selection criteria for controls was absence of PD and systemic disease.

Table 4. The number of subjects in RA and PD groups for studies II, III and IV

Patients with RA	Total	Females	Males
<i>Study II and III</i>	38	33	5
<i>Study IV</i>	47	41	6
Patients with PD only	Total	Females	Males
<i>Study II and III</i>	38	26	12
<i>Study IV</i>	51	33	18

Cells

For *study I*, human blood lymphocytes from healthy volunteers at Huddinge University Hospital were used after purification using Lymphoprep. Monocytes and phagocytic cells were treated with carbonyl iron and removed magnetically. The use of lymphocytes from the blood of healthy individuals was approved by the local ethics committee. The cell preparations consisted of 82–93% CD3-positive cells. T cells were further enriched and purified by depleting CD56-, CD19- and CD14- positive cells using beads coated with the corresponding antibodies. Lymphocytes were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 0.16% sodium bicarbonate, 10,000 U/ml benzylpenicillin, 10,000 μ g/ml streptomycin and 10% fetal calf serum or in serum-free AIM-V medium. The experiments

were performed under serum-free conditions in order to exclude any interference of exogenous proteins and peptides.

QUESTIONNAIRE

For *study II, III* and *IV*, a questionnaire was used to collect information from participating subjects regarding presence of self-reported chronic diseases and previous hospitalization history. Detailed information regarding oral health status, past dental history, oral hygiene measures, use of medication and smoking habits were recorded. Medical records and prescription for medication was requested for verifying self-reported conditions.

PERIODONTAL EXAMINATION

A detailed periodontal examination was carried out for all participating subjects in *study II, III* and *IV* at the Department of Periodontology, Altamash institute of Dental Medicine, Karachi, Pakistan. The examination comprised of recording plaque index (PI), bleeding on probing (BOP) and periodontal probing depth (PPD) for all teeth excluding third molars. The indices recorded four sites per tooth. Periodontal disease was defined as three sites or more in three different teeth with PPD of $\geq 5\text{mm}$. For pocket measurement, Hu Friedy's Goldman-Fox probe was used. BOP was recorded as present or absent within 30 seconds of probing. The scores were recorded as a mean percentage for PI and BOP and as number of pockets measuring PPD 3- $<5\text{mm}$ and PPD $\geq 5\text{mm}$. For *study IV*, the total cumulative pocket depth was used as a continuous variable. The number of missing teeth was also recorded.

Embedded root remnants of teeth were considered as missing.

Additionally, a novel index was derived by adding the probing pocket depth measurements from all measured sites to give a total aggregate whose value is the sum of each of the PPD site measurement in millimeters. All pockets measuring 5mm or more were added separately to calculate of sum of deep pockets representing diseased sites. The collective sum of PPD was further divided by the total number of teeth to provide a tooth adjusted ratio.

$$\sum PPD \text{ Total} = \text{sum of all probing pocket depths (mm)}$$

$$\sum PPD \text{ Disease} = \text{sum of probing pocket depths} \geq 5\text{mm}$$

$$\text{Tooth - adjusted PPD Total} = \frac{\sum PPD \text{ Total}}{\text{Number of teeth}}$$

RADIOGRAPHIC EVALUATION

Radiographic evaluation was performed for *studies II, III* and *IV* to quantify marginal bone loss (MBL). Panoramic radiographs were taken for all study subjects using a digital extra oral tomography machine (SironaOrthophos 3, Germany) at the Department of Periodontology, Altamash institute of Dental Medicine, Karachi, Pakistan. All radiographs were measured digitally on a computer screen using SIDEXIS software. One pixel was equal to 0.09mm. The assessment for MBL was made as a vertical measurement starting from the cemento-enamel junction (CEJ) and terminating at the most apical portion of the marginal bone. MBL was measured for premolars and molars (excluding third molars). An average value for MBL per tooth was calculated after taking two readings of mesial and distal sides each on digital

radiographs. Teeth at which the CEJ and bone crest were not clear because of technical issues such as overlapping restorations or dental caries were excluded.

DAS28

The DAS28 is a Disease Activity Score based on 28 joints for RA which is used to make a reproducible and comparable assessment of the rheumatoid arthritis activity (Prevoo et al., 1995). DAS28 scoring was used in *study III* and *IV* for all RA subjects, calculated by the referring rheumatologist. DAS28 scoring is based on the following: number of tender joints, number of swollen joints, visual analogue scale score of the patient's global health, and erythrocyte sedimentation rate (ESR). Remission was considered for a score between 0 and <2.6. Low activity corresponded to 2.6 to <3.2 whereas moderate activity was between 3.2 and ≤ 5.1. High activity was strictly above 5.1.

BLOOD SAMPLE

Peripheral venous blood via venipuncture was collected from all subjects. The samples were collected in 4 ml serum tubes without any additives (Vacutainer tubes, BD Biosciences, USA). The blood samples were allowed to coagulate and then centrifuged at 1790 x *g* for 10 minutes. Post- centrifugation, the serum was transferred to 2ml storage tubes and were kept frozen at -22°C in Pakistan until their transportation to Sweden where they were stored at -80°C.

SEROLOGICAL ANALYSES

IgM RF and ESR

For *study II, III* and *IV*, IgM-RF levels and ESR were measured for all RA patients. For quantification of rheumatoid factor (RF), turbidimetric reagents were used in an automated clinical chemistry system. The reagent Quantia RF 6K44-01 (Abbott Diagnostics, Illinois, USA) comprises of R1 (activation buffer) and R2 which is a suspension of polystyrene latex particles of uniform size coated with human gamma globulin. The sample was mixed with reagent R1 and R2, after which agglutination occurs. This was then measured by turbidimetry allowing quantitative determination of IgM-RF using Abbott ARCHITECT c8000 system (Abbott Diagnostics). Results are expressed in IU/mL based on the WHO standard (Anderson et al., 1970). The system was closely monitored. Both high and low controls were run with every batch analyzed. The within-run CV was 0.6% at concentration 54.8 IU/ml and 0.7% at 119.9 IU/ml. The levels of RF < 30 U/ml were defined normal, 30 - 50 U/ml as low level positive and values > 50 U/ml as high-level positive. ESR was estimated by using the Westergren method manually (normal range: 0 to 20 mm per hour).

Anti-cyclic citrullinated peptide (anti-CCP) antibodies

For *study II*, anti-CCP antibodies were measured for all subjects. BioPlex®2200 immunoassay was used to analyze the anti-CCP levels in the serum samples (Bio-Plex™ 2200 anti-CCP, Bio-Rad Laboratories, Hercules, CA, USA). The principle of this immunoassay method is based on the heterogeneous sets of magnetic beads allowing semi-quantitative detection of IgG antibodies to cyclic citrullinated peptide (CCP) in serum. In this system, the patient sample aliquot, sample diluent, and bead reagent are combined into a reaction vessel and incubated at 37°C. After a wash cycle, unbound antibodies are removed and anti-human IgG conjugated to phycoerythrin is added to the mixture which is incubated

at 37°C. Excess conjugate is removed in another cycle and the washed beads are re-suspended on wash buffer. The bead mixture is then passed through the detector and the assay identity is determined by the fluorescence embedded in the surface of the bead. The amount of immobilized antibody is determined by the fluorescence of the anti-IgG reporter conjugate. Raw data are collected in relative fluorescence intensity (RFI). The RFI is converted to U/mL using the calibration curve established by the six levels of BioPlex®2200 Anti-CCP Calibrators. All the samples were run as singletons.

HbA1C

Glycated hemoglobin levels were determined for all subjects in *study IV*. Blood was drawn in 4 ml collection tubes with EDTA (Vacutainer tubes, BD Biosciences, USA). The tubes were submitted on the same day for HbA1c analyses at Karachi Laboratory Diagnostic Centre, Karachi, Pakistan. For quantification of HbA1c, a Hemoglobin A1c program based on ion-exchange high-performance liquid chromatography was performed using the Bio-Rad D-10 Hemoglobin Testing System (Bio-Rad Laboratories, Hercules, CA, USA).

Multiplex PEA-based immunoassay

The proximity extension assay (PEA) is a homogenous immunoassay which is based on 94 pairs of specific antibodies that are equipped with oligonucleotides (PEA probes) and mixed with an antigen-containing sample. Upon sample incubation, all proximity probe pairs bind their specific antigens, which brings the probe oligonucleotides in close proximity to hybridize. The oligonucleotides have unique annealing sites that allows pair-wise binding of matching probes. Addition of a DNA polymerase leads to an extension and joining of the two oligonucleotides and formation of a PCR template. Universal primers are utilized to pre-amplify all 96 different DNA templates in parallel. Uracil-DNA glycosylase partly digests the DNA templates and remove all unbound primers. Finally, each individual DNA sequence is detected and quantified using specific primers in by microfluidic qPCR.

Proseek Multiplex Inflammation I (Olink Bioscience, Uppsala, Sweden), was used for conducting PEA, according to the manufacturer's instructions. The panel simultaneously measures 92 biomarkers, as a homogeneous assay, in a 96-well microtiter plate format. The samples were assayed as singletons. One microliter (µl) sample was mixed with 3 µl incubation mix that contained pairs of probes (each consisting of a DNA oligonucleotide labelled). This mixture was incubated at 8 °C overnight, followed by addition of 96 µl extension mix containing PEA enzyme and PCR reagents which were added and incubated for 5 min at room temperature before transferring the plate to a thermal cycler. In the cycler, the plate underwent 17 cycles of DNA amplification. A 96.96 Dynamic Array IFC (Fluidigm, South San Francisco, CA, USA) was prepared and primed according to the manufacturer's instructions. Using 7.2 µl detection mix and mixing it with 2.8 µl of sample mixture in a new plate, 5 µl of this was loaded into the right side of the primed 96.96 Dynamic Array IFC. On the left side of the 96.96 Dynamic Array IFC, the unique primer pairs for each cytokine were loaded and the protein expression program was run in Fluidigm Biomark reader, in accordance with the instructions. Details regarding data validation, limit of detection (LOD), specificity and reproducibility are available via Olink's website (<http://www.olink.com/data-you-can-trust/validation/>). Calibrator curves for correlating the normalized protein expression (NPX) values with actual concentrations can also be found in Olink's website (<http://www.olink.com/proseek-multiplex/inflammation/biomarkers/>). The assays were performed blinded without knowledge of clinical data.

ELISA

For *study I*, 96-well polystyrene plates were used which were coated with 0.25 µg/ well of RAP overnight in coating buffer (1.59 g Na₂CO₃, 2.93 g NaHCO₃, 200 mg NaN₃) at 4°. Overnight and incubated with medium samples for 1 hr at 4°. Bound LRP1 was detected using anti-LRP1 antibody 0.001 µg/ml (1 hr; 4°), rabbit anti-mouse diluted 1:1000 (1 hr; 4°), and a sheep anti-rabbit immunoglobulin diluted 1 : 4000 (1 hr; 4°).

For *study III*, Human Osteopontin (OPN), TNF R1, TNF R2 and RANKL were analyzed using commercial sandwich ELISAs (DY1433, DY225, DY726 and DY626, R&D Systems, Minneapolis, MN, USA). The assays utilized a monoclonal antibody which was specific for each peptide and coated onto separate microtiter plates. Standards were assayed as duplicates and samples were assayed as singletons. They were pipetted into the wells and the peptides were bound to the immobilized antibodies. After washing, a biotinylated antibody was added. After another incubation and washing cycle, a streptavidine-HRP conjugate was added to the wells followed by further incubation and washing. This was followed by the addition of a substrate solution. The development was stopped and the absorbance was measured in a SpectraMax 250 (Molecular Devices, Sunnyvale, CA, USA). The peptide concentrations in the samples were determined by comparing the optical density of the sample with the standard curve. The coefficient of variation for the ELISAs were approximately 6%.

QUANTITATIVE IMMUNOCYTOCHEMISTRY

The expression of cell surface antigens was analyzed in cells fixed in 2% paraformaldehyde at 4° attached to glass slides coated with poly-L-lysine (PLL), intercellular adhesion molecule 1 (ICAM-1) or fibronectin at 4° overnight. Antigen expression was detected with monoclonal antibodies and a complex of biotinylated peroxidase and avidin (Vector Laboratories, Burlingame, CA). For detection of intracellular antigens cells were fixed in 2% paraformaldehyde and permeabilized by 0.1% saponin. The cells were examined in a Nikon Eclipse E1000M microscope. The intensity of the immunocytochemical staining was quantified using the image processing and analysis program IMAGEJ.

SMALL INTERFERING RNA-MEDIATED GENE SILENCING

The expression of LRP1 was suppressed using the human T-cell Nucleofactor kit (Lonza, Köln, Germany) and a Nucleofactor device (Amaxa biosystems, Köln, Germany). Briefly, 5 x 10⁶ T enriched cells were resuspended in 100 µl of nucleofactor solution and transfected with 500 nM final concentration of small interfering RNA (siRNA) using protocol U14. The siRNA consisted of TSP-1 siRNA (human) (Alternative 1) (A:Sense: CCACGAUGAUGACAACGAUtt. Antisense: AUCGUUGUCAUCAUCGUGGtt. B: Sense: CGAGACGAUUGUAUGAAGAtt. Antisense: UCUUCAUACAAUCGUCUCGtt. C: Sense: GAAGAAGCGUAAAGACUAUtt. Antisense: AUAGUCUUUACGCUUCUUCtt), LRP1 siRNA (Alternative 1) (human). Sense: AAGACUUGCAGCCCCAAGCAGtt. Antisense: CUGCUUGGGGCUGCAAGUCUtt) and control siRNA (sc-37007) from Santa Cruz Biotechnology (Dallas, TX) delivered by AH diagnostics, Skärholmen, Sweden. TSP-1 siRNA Suppl (human): (Sense: GCAUGACCCUCGUCACAUAtt. Antisense: UAUGUGACGAGGGUCAUGCca) and LRP1 SiRNA Suppl (human) (Sense: GCUGUGACAUGGACCAGUUtt. Antisense: AACUGGUCCAUGUCACAGCgg) were obtained from Applied Biosystems (Stockholm, Sweden). The degree of gene silencing and the influence of silencing on adhesion and activation were determined 40 hr after introducing siRNAs.

BIOTINYLATION AND IMMUNOPRECIPITATION

The surface membrane of lymphocytes was labelled with D-biotinyl-e-aminocaproic acid-N-hydroxysuccinimide ester (biotin-7-NHS) according to the manufacturer's description (Roche Diagnostics GmbH, Germany). For immunoprecipitation, adherent cells were first biotinylated, released by a cell scraper and then immunoprecipitated. Cells which were in suspension and non-adherent cells were biotinylated and immunoprecipitated directly. A 75 μ l stop solution per tube was used to stop the reaction after incubation for 15 min at room temperature and centrifuged at 490 *g* for 10 min. After discarding the supernatant, 5 ml cold PBS was added to each tube followed by centrifugation at 490 *g* for 10 min. Using 1 ml lysis buffer (50 mM core buffer, 150 mM NaCl, 0.1 mg/ml PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1% Nonidet P-40 and 0.5% sodium deoxycholate), the cells were lysed and incubated for 30 min on ice. The cells were resuspended after incubation and centrifuged at 12 000 *g* for 10 min at 4° after which the supernatants were transferred to clean Eppendorf tubes. Immunoprecipitation was carried out with protein G agarose beads according to the manufacturer's instructions (Roche). The supernatants were mixed with 1 μ g antibody at 4° overnight followed by centrifugation at 12 000 *g* at 4° for 20 seconds. Subsequently, the supernatants were discarded and the beads were re-suspended in 1 ml washing buffer, and centrifuged again at 12 000 *g* at 4° for 20 seconds, the same procedure was repeated twice. After washing, 20 μ l reducing buffer (2 x, containing 0.15 g dithiothreitol in 5 ml immunoprecipitation buffer (150mM NaCl, 10 mM Tris-HCL pH 7.4)) was mixed with the beads and heated at 95° for 4 min and subsequently centrifuged at 7000 *g* for 1 min to spin down the beads and the proteins were separated on SDS–PAGE gels. Proteins were transferred to the Hybond ECL membrane (Amersham, Chalfont St Giles, UK) and detected using the BMC chemiluminescence blotting kit (Roche).

Western blotting

After separation of the samples on SDS–PAGE gels, they were blotted onto a nitrocellulose membrane (Amersham) and blocked over night with PBS, 4% BSA, and 0.5% Tween. Filters were washed with PBS with 1.5% BSA and incubated with antibodies. ECL Western blotting detection reagents were used for detection with Hyperfilm TM (Amersham).

Cell motility

Collagen type 1 was first diluted in serum-free RPMI-1640 and H₂O (8/1/1) after which it was applied in plastic Petri dishes 1 ml/dish (30 mm; BD Biosciences, Franklin Lakes, NJ). It was then allowed to polymerize at room temperature. Using AIM-V medium, a total of 1.0 x 10⁶ cells was added to each well with and without antibodies and allowed to migrate for different times. The cells were fixed in 2.5% glutaraldehyde for 10 min or in 2% paraformaldehyde for 20 min for immunocytochemistry and washed twice with PBS. Cell morphology and cell migration were evaluated in nine fixed positions in each well. An inverted microscope (Nikon Eclipse TE300) and a digital depth meter (Heidenheim ND221) were used to study cell morphology and migration at 50- μ m intervals throughout the gel. The mean number of infiltrating cells/field (x 20 objective) per infiltration depth (50 μ m for the first two layers immediately beneath the gel surface and 100 μ m for other layers further down) were recorded as results. After fixation in paraformaldehyde, immunocytochemistry was used to identify the infiltrating cells in situ in the collagen gels. Migration was also analysed in a modified Boyden assay (transwell assay) using 8- μ m nucleopore filters coated with ICAM-1 (2 μ g/ml) or fibronectin (10 μ g/ml). The lower wells of 48-well Boyden

chambers were filled with RPMI containing 1 mg/ml BSA and CXCL12 (50 ng/ml) whereupon the coated filters were placed in the chambers. The upper chambers were filled with 50 µl of 2×10^6 cells/ml in AIM-V. Following incubation for 1 hr, the number of cells in the lower chamber was counted in triplicate.

Cell adhesion

In order to study cell adhesion, plastic Petri dishes (90 mm; Heger A/S, Rjukan, Norway) were coated with ICAM-1 (2 µg/ml), poly-L-lysine (10 µg/ml) or fibronectin (10 µg/ml), and extensively washed before use. Using AIM-V medium, the cells (10 000/position) were incubated on the substrates for different times, then fixed in 2.4% cold glutaraldehyde for 10 min for immunocytochemistry and unbound cells were removed by gentle aspiration. The number of adherent cells per microscope field (20 x objective) was counted.

T-cell activation

T cell activation was achieved using mixed lymphocyte culture activation. T cells were co-cultured with inactivated allogeneic stimulator cells whereupon interferon- γ (IFN- γ) and interleukin-2 (IL-2) production was determined by an ELISPOT assay. T-cell activation was also accomplished using anti-CD3. T cells were cultured on a surface coated with anti-CD3 antibodies with and without CXCL12.

STATISTICAL ANALYSES

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 21.0 (SPSS Inc, Chicago, IL, USA). The significance level was set at 5%.

In *study I*, parametric tests were used as staining intensity in immunocytochemistry experiments, number of migrating cells and adherent cells were presented as mean \pm SD in arbitrary units. For evaluating differences between groups, the Mann–Whitney *U* test was used. For determination of differences in migration assays, paired Student's *t*-test was used.

In *study II*, parametric tests were used to calculate the differences in periodontal parameters between the study groups. One-way ANOVA was used for this comparison. Tukey's HSD (honest significant difference) test was used in conjunction with one-way ANOVA to correct for family-wise error rate that can occur during multiple comparisons. For biomarker analyses, the data was normalized and generated as Normalized Protein eXpression (NPX) unit on a log₂ scale and reported in NPX units. A larger NPX number represents a higher protein level in the sample, with the background level at around zero. This was done using a Wizard generated by Olink, together with the statistical software GenEx. The assay generated a delta C_q (dcq) value for each data point via Olink Wizard with GenEx software thus normalizing each sample for technical variation in one run. This was followed by normalization between runs through subtraction of the interplate control (IPC) for each assay. For the final step, the values were set relative to a fixed correction factor which is determined by Olink. Non-parametric Spearman's rank correlation was performed to analyze the associations between cytokine levels and clinical parameters, respectively. To reduce the risk of false discoveries due to multiple testing, the Benjamini-Hochberg false discovery rate method was used to adjust the *p*-values.

For *study III*, non-parametric statistics were used as data are reported as medians and interquartile ranges. Group wise comparison for analyzing the variables was performed using the Mann–Whitney *U* test. Spearman rho coefficient was used to correlate between markers in the study groups and clinical parameters. To reduce the risk of false discoveries, the Benjamini-Hochberg false discovery rate method was used for adjusting values.

In *study IV*, data are reported as medians and interquartile ranges. For group wise comparison, the Mann–Whitney *U* test was used. Spearman rho coefficient was used to correlate between variables in the study groups.

In *studies II, III* and *IV*, all analyses involving MBL were performed using data for mandibular MBL as a high proportion of MBL measurements in maxillary premolars and molars were not possible. This was due to difficulty in determining the alveolar bone crest.

RESULTS

STUDY I

As part of maintaining important immunological patrolling and immuno-surveillance functions, T cells require the ability to be motile, adhesive, and activated upon signaling. We wanted to demonstrate whether these characteristics may depend on the large transmembrane cell surface receptor low-density lipoprotein receptor-related protein 1 (LRP1) and its ligand thrombospondin-1 (TSP-1). LRP1 consists of an α -chain (515 kDa) containing ligand-binding domains, a β -chain (85 kDa) containing the transmembrane domain and the cytoplasmic tail, and has binding sites for more than 40 ligands. TSP1 is a 450-kDa glycoprotein composed of three identical disulfide-linked polypeptide chains that display binding sites for various cell surface receptors. LRP1 mediates the endocytosis of a vast array of distinct ligands, including proteases, protease inhibitor complexes, extracellular matrix proteins and growth factors. It also regulates the cell surface abundance of other membrane proteins, thereby regulating cell signaling activity (Strickland et al., 2002). We found that co-stimulation through CD28, integrins such as ICAM-1 and fibronectin, and CXCR4 inhibits a protease mechanism that removes LRP1 from the cell surface. This removal or 'shedding' of LRP1 antagonizes TCR-induced activation.

A broad spectrum metalloprotease inhibitor stimulates cell surface expression of LRP1 and TSP-1 expression

Adhesion experiments in the presence of broad spectrum MMP inhibitor GM6001 showed that cells attached on ICAM-1 and fibronectin had pronounced surface expression of LRP1 and TSP-1 ($P < 0.01$) compared to those without. This increase was seen in both non-stimulated and activated cells. Contact with fibronectin and ICAM-1 also enhanced LRP1 expression, although not as strongly as in the presence of GM6001.). The shedding of LRP1 is brought about by sheddases including ADAM10 (Shackleton et al., 2016). Sheddases are active proteases from the ADAM (a disintegrin and metalloproteinase) membrane-bound enzymes that cleave extracellular portions of proteins from the cell surface. ADAM10 is a sheddase that might be responsible for the shedding of LRP1 in non-lymphoid cells, and represent a general shedding machinery for membrane proteins. LRP1 was detectable in the culture medium of T cells and that GM6001 (10 μ M) and the ADAM10 inhibitor GI254023X (5 μ M) reduced the appearance of LRP1 in the medium.

Comparison of the cell surface expression of TSP-1 induced by adhesion with the total cellular TSP-1 before adhesion using Western blotting showed that T cells before adhesion exhibited negligible surface expression of TSP-1. Adhesion to ICAM-1, triggered surface expression of 170 000 and 130 000 bands precipitated with an antibody to TSP-1. In cells incubated in the presence of GM6001, anti-TSP-1 precipitated a 170 000 (full length TSP-1) band and an almost negligible 500 000 band (LRP1). In cells on ICAM-1, anti-LRP1 precipitated a prominent 500 000 band and relatively weak 170 000 and 130 000 bands. In cells incubated on ICAM-1 in the presence of GM6001, anti-LRP1 precipitated a very prominent 500 000 band and prominent 170 000 and 130 000 bands, whereas anti-TSP-1 precipitated very prominent 170 000 and 130 000 bands and a prominent 500 000 band. Western blotting of cells incubated on ICAM-1 in the presence of GM6001 confirmed that the 500 000 band is LRP1 and that the 170 000 and 130 000 bands are TSP-1. This Western blot also confirmed that anti-LRP1 co-precipitated TSP170 and TSP130, whereas anti-TSP-1 co-precipitated LRP1.

The reactivity of the anti-LRP1 and anti-TSP-1 antibodies also verified that the presence of GM6001 preferentially stimulates LRP1 expression, whereas ICAM-1 stimulates both LRP1 and TSP-1 expression.

Co-stimulation enhances surface expression of LRP1

T cells were incubated in the presence of exogenous TSP-1 to see their effect on LRP1 expression. This TSP-1, but not IgG, was indeed found to prevent shedding of LRP1. We next examined whether the enhancing effect of ligation of CD28 (anti-mouse IgG, 2 µg/ml + anti-CD28, 50 ng/ml) on LRP1 expression was dependent on shedding. This experiment showed that CD28 ligation inhibited the appearance of LRP1 in the medium and increased cell surface LRP1.

TSP-1 mediates LRP1-dependent adhesion to ICAM-1 and fibronectin

To elucidate whether the stimulation of T-cell adhesion to fibronectin and ICAM-1 in the presence of GM6001 and GI252023X was dependent on LRP1 and/ or TSP-1, siRNA was used to silence these molecules. The siRNA results showed that LRP1 siRNA as well as TSP-1 siRNA markedly reduced TCR-induced T-cell activation. LRP1 siRNA increased the cell surface expression of TSP-1. This indicates that an increased cell surface expression of TSP-1 accounts for the enhancement of adhesion by LRP1 siRNA.

GM6001 and CXCL12 enhance TCR-induced T-cell activation through TSP-1 and LRP1

By examining the influence of knockdown of TSP-1 and LRP1 on T-cell activation by anti-CD3 (50 ng/ml) in the presence of CXCL12, we observed that TSP-1 siRNA inhibited anti-CD3-induced IFN- γ production as well as the enhancing effect of CXCL12. Control siRNA did not inhibit IFN- γ production. LRP1 siRNA had a relatively weak albeit significant inhibitory effect whereas a combination of TSP-1 siRNA and LRP siRNA had a pronounced inhibitory effect.

STUDY II

Characteristics of Study Subjects

The characteristics of all the study subjects are presented in Tables 5a.

Table 5a. Characteristics of Study Group

Total subjects (n)	90
Mean age distribution in years (range)	46.4 ± 10.1 (21 – 70)
Females (n, %)	64 (71)
Total subjects with RA (n)	38
Subjects with RA and PD (n, %)	19 (50)
Subjects with RA and without PD (n, %)	19 (50)
Total subjects with PD only (n)	38
Healthy Controls (n)	14

RA=rheumatoid arthritis PD= periodontal disease.

The characteristics for the RA groups (with and without PD) are presented in table 5b. On comparison, the IgM-RF levels were higher in the RA with PD group. All remaining rheumatological characteristics were comparable between the two groups.

Table 5b. Characteristics of the rheumatoid arthritis (RA) group

Characteristic	RA without PD (n=19)	RA with PD (n=19)	P-value
Years since diagnosis	8.7	8.1	0.76
IgM-RF (≥30 IU/mL)	117.5	168.7	0.04
ESR (mm/hr)	49.8	54.8	0.15
Anti-CCP (≥ 3 U/mL)	251.7	286.1	0.62

Values are shown as mean ± SD.

IU = international units

Anti-CCP values are expressed as arbitrary units (U/ml)

PD= periodontal disease

Biomarkers

The inclusion of biomarkers for analytical purpose was based on the criteria that $\geq 60\%$ of subjects should have detectable levels for the given biomarker. According to this criteria, a total of 66 cytokines qualified out of 92 which are shown in Table 6.

Table 6. Biomarkers with $\geq 60\%$ of results included in the analyses

Adenosine Deaminase (ADA)	Interleukin-10 receptor subunit alpha (IL-10RA)
Beta-nerve growth factor (Beta-NGF)	Interleukin-10 receptor subunit beta (IL-10RB)
Caspase 8 (CASP-8)	Interleukin-12 subunit beta (IL-12B)
C-C motif chemokine 2 (CCL2) "MCP-1"*	Interleukin-15 receptor subunit alpha (IL-15RA)
C-C motif chemokine 3 (CCL3) " MIP-1 alpha"	Interleukin-18 (IL-18)
C-C motif chemokine 4 (CCL4)	Interleukin-18 receptor 1 (IL-18R1)
C-C motif chemokine 7 (CCL7) "MCP-3"	Latency-associated peptide transforming growth factor beta 1 (LAP TGF-beta-1)
C-C motif chemokine 8 (CCL8) "MCP-2"	Leukemia inhibitory factor receptor (LIF-R)
C-C motif chemokine 11 (CCL11) "Eotaxin"	Macrophage colony-stimulating factor 1 (CSF-1)
C-C motif chemokine 13 (CCL13) "MCP-4"	Matrix metalloproteinase-1 (MMP-1)
C-C motif chemokine 19 (CCL19)	Matrix metalloproteinase-10 (MMP-10)
C-C motif chemokine 20 (CCL20)	Natural killer cell receptor 2B4 (CD244)
C-C motif chemokine 23 (CCL23)	Neurotrophin-3 (NT-3)
C-C motif chemokine 25 (CCL25)	Oncostatin-M (OSM)
C-C motif chemokine 28 (CCL28)	Osteoprotegerin (OPG)
CD40L receptor (CD40)	Programmed cell death 1 ligand 1 (PD-L1)
CUB domain-containing protein 1 (CDCP1)	Protein S100-A12 (EN-RAGE)
C-X-C motif chemokine 1 (CXCL1)	Signaling lymphocytic activation molecule (SLAMF1)
C-X-C motif chemokine 5 (CXCL5)	SIR2-like protein 2 (SIRT2)
C-X-C motif chemokine 6 (CXCL6)	STAM-binding protein (STAMPB)
C-X-C motif chemokine 8 (CXCL8) "IL-8"	Stem cell factor (SCF)
C-X-C motif chemokine 9 (CXCL9)	Sulfotransferase 1A1 (ST1A1)
C-X-C motif chemokine 10 (CXCL10)	T-cell surface glycoprotein CD5 (CD5)
C-X-C motif chemokine 11 (CXCL11)	T cell surface glycoprotein CD6 isoform (CD6)
C-X ₃ C motif chemokine ligand 1(CX3CL1) " Fractalkine"	TNF-beta (TNFB)
Cystatin D (CST5)	TNF-related activation-induced cytokine (TRANCE)
Delta and Notch-like epidermal growth factor-related receptor (DNER)	TNF-related apoptosis-inducing ligand (TRAIL)
Fibroblast growth factor 19 (FGF-19)	Transforming growth factor alpha (TGF-alpha)
Fms-related tyrosine kinase 3 ligand (Flt3L)	Tumor necrosis factor (Ligand) superfamily, member 12 (TWEAK)
Hepatocyte growth factor (HGF)	Tumor necrosis factor ligand superfamily member 14 (TNFSF14)
Interleukin-6 (IL-6)	Tumor necrosis factor receptor superfamily member 9 (TNFRSF9)
Interleukin-7 (IL-7)	Urokinase-type plasminogen activator (uPA)
Interleukin-10 (IL-10)	Vascular endothelial growth factor A (VEGF-A)

*Common synonyms used in Olink inflammation Panel (Panzai et al., 2017).

Periodontal Parameters for study groups.

Results for comparison of periodontal parameters between healthy, RA (with and without PD) and PD groups are shown in Table 7. The number of pockets measuring 3- <5mm did not differ between the PD and RA disease groups. Mean values for MBL, BOP and PPD \geq 5mm were the highest in the PD group. The number of teeth were comparable between all four groups.

Table 7. Clinical (BOP, PPD 3-<5mm, PPD \geq 5mm, number of teeth) and radiographic (mandibular MBL) parameters in RA (with and without PD), PD and healthy groups

	PD (n=38)	RA with PD (n= 19)	RA without PD (n= 19)	Healthy (n=14)
BOP%	76.2 \pm 27*‡§	37 \pm 32	42.6 \pm 31‡	27.2 \pm 29.4
PPD 3- <5mm	49.2 \pm 17.4§	43.5 \pm 17.9**	55.5 \pm 16.2§	26.9 \pm 14.1*
PPD\geq 5mm	33.4 \pm 14.1*‡§	18.2 \pm 15.2§	0.32 \pm 0.74‡	0.4 \pm 0.5
Number of Teeth	24.8 \pm 4.3	25.5 \pm 4.1	26.8 \pm 2.9	26.1 \pm 4.7
Mandibular MBL mm	5.4 \pm 2.6*‡§	3.8 \pm 2.2	2.9 \pm 1	3.3 \pm 0.7

Differences in the means \pm SD of clinical parameters were tested using one-way ANOVA. For multiple comparisons, post- hoc Tukey HSD test was performed.

* compared to RA without PD. P<0.01

‡ compared RA with PD. P<0.01

§ compared to healthy controls. P<0.01

** compared to healthy controls. P<0.05

BOP = Bleeding on probing, PPD= Probing pocket depth, MBL= marginal bone loss

RA=rheumatoid arthritis PD= periodontal disease

Correlation of cytokines with periodontal parameters

All correlations determined between five periodontal parameters and 66 different cytokines (66 \times 5) are shown in Figure 6. The periodontal parameters comprised of shallow (measuring 3-<5mm) and deep (measuring \geq 5mm) pockets, BOP, mandibular MBL and number of teeth which were used to identify associations with cytokine levels using Spearman rank. RA subjects with PD were excluded in order to avoid positive or negative confounding and the analyses was conducted for a singular cohort of 69 subjects. All cytokines were found to correlate negatively with the number of shallow pockets except for CD40. For BOP, five cytokines were positively correlated, while the remaining five were inversely correlated. The number of deep pockets was significantly correlated with a total of 18 cytokines out of which eight were positively and ten were inversely correlated. A total of seventeen cytokines correlated significantly with MBL. A higher frequency of significant correlations was seen for number of teeth which associated with 27 cytokines out of which only five were negative coefficients.

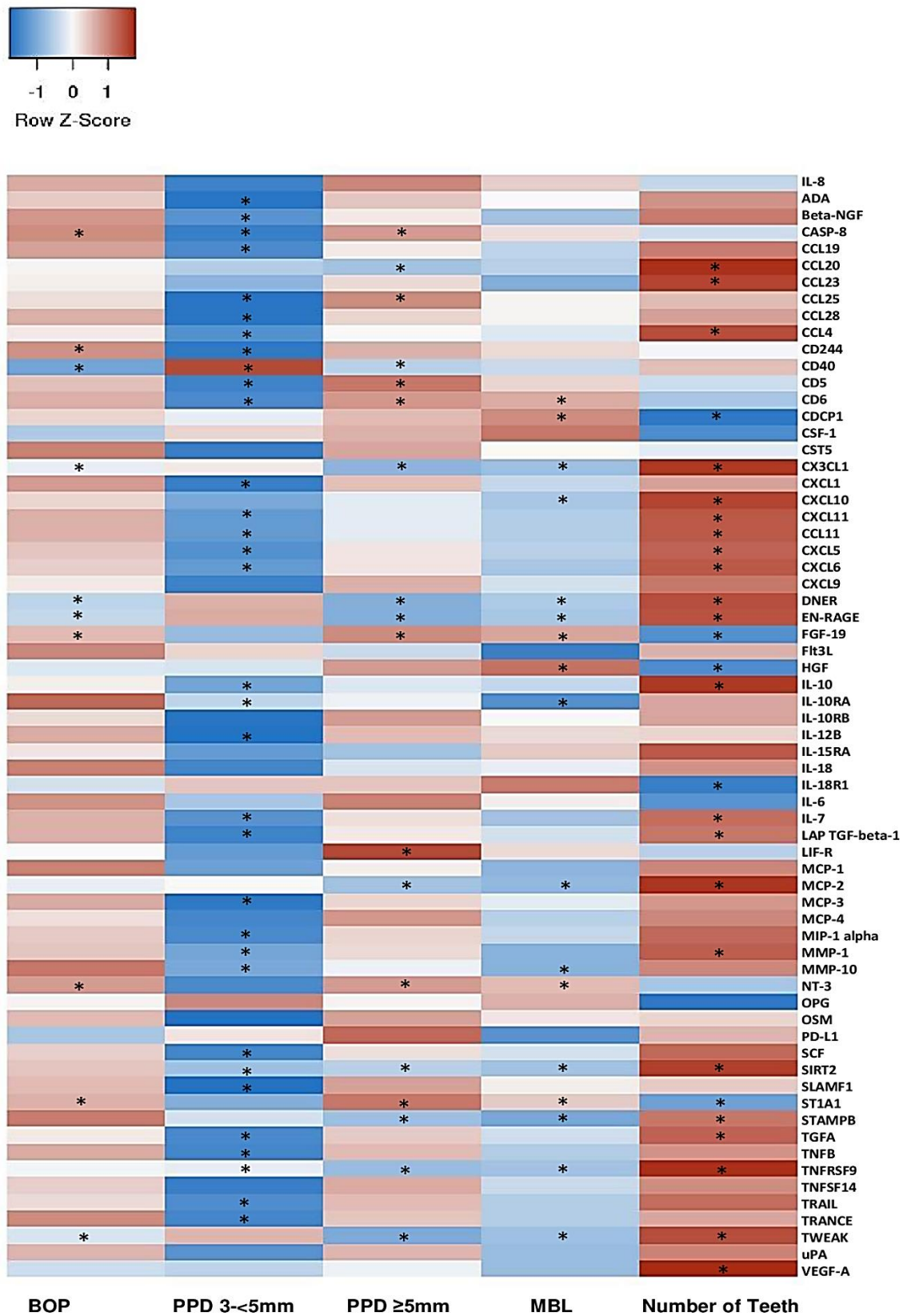


Figure 6. Heatmap showing Spearman correlation between clinical parameters and 66 different cytokines (66 × 5) for a singular cohort of 69 subjects.

*P < 0.05

Subgroup analysis for correlation of cytokines in RA subjects

In order to compare which cytokines correlated with periodontal variables under the influence of periodontal disease, a subgroup analysis was performed for RA subjects. Results for significant correlations are shown in Figures 7 and 8. In RA without PD subjects, MBL was found to correlate positively with SLAMF1, CST5 and ADA whereas number of teeth were correlated with DNER. Shallow pockets (PPD 3-<5mm) were found to inversely correlate with MMP-10 and TGFA whereas BOP with ADA and SLAMF1 respectively.

In RA with PD subjects, Flt3L and EN-RAGE with BOP and MCP-3 with MBL were the only two markers to correlate positively.

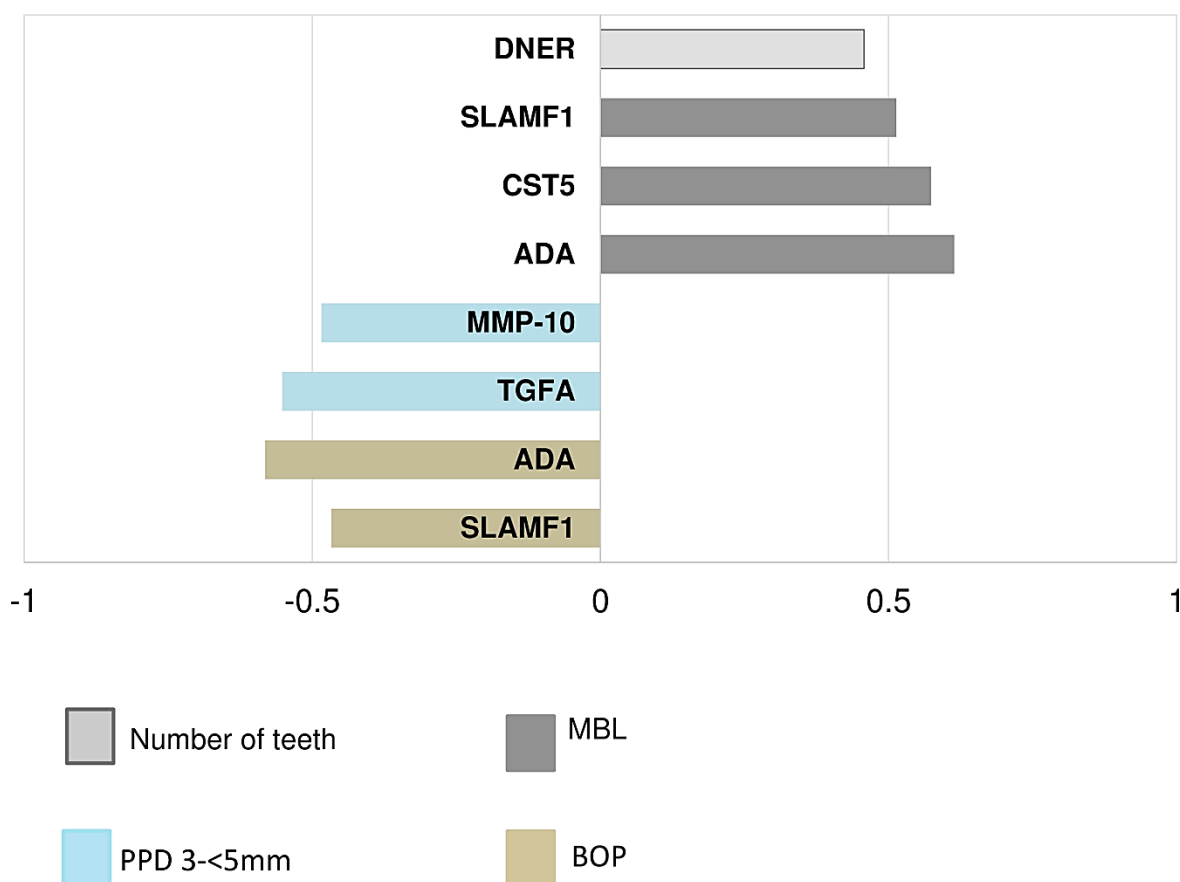


Figure 7. Spearman rank correlations between cytokines and clinical parameters for RA group without PD (n=19). The x axis represents Spearman r coefficient. All correlations shown are significant ($P < 0.05$).

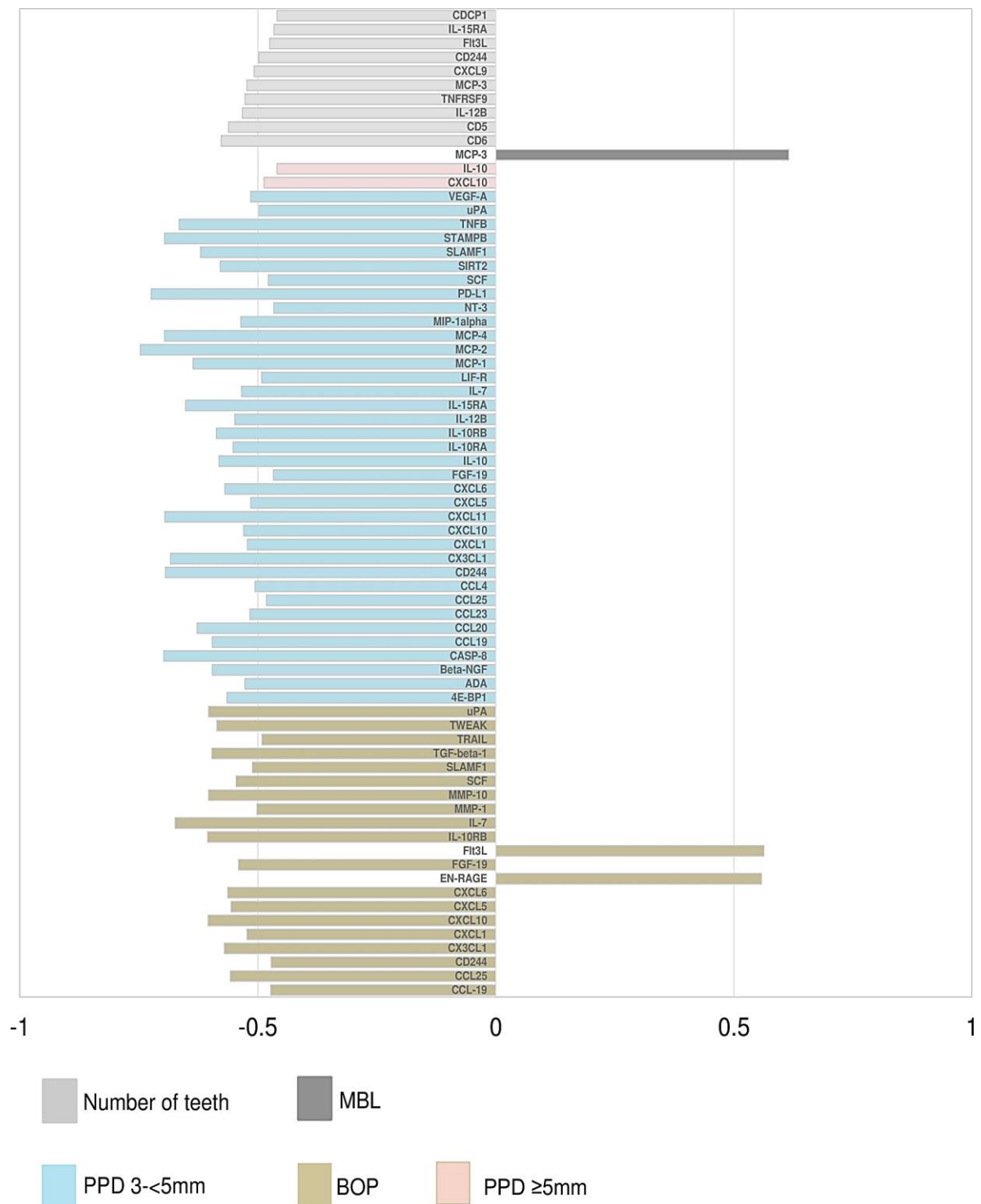


Figure 8. Spearman rank correlations between cytokines and clinical parameters for RA group with PD (n=19). The x axis represents Spearman r coefficient. All correlations shown are significant (P < 0.05).

STUDY III

General characteristics for all study subjects

A total of 90 subjects participated in this study. The mean age was 46.4 years. The total number of subjects diagnosed with PD was 57 (with RA = 19, without RA= 38) while those without PD were 33 (with RA = 19, healthy = 14). For comparative analyses, the subjects were further divided into four groups: subjects with PD only, RA subjects with and without PD and healthy groups.

Correlation between clinical and serological data

Serum RANKL was found to associate with number of PPD \geq 5mm ($r=0.28$, $P=0.01$) and MBL ($r = 0.29$, $P=0.008$). Inverse correlations were found between OPN and the number of PPD 3-<5mm ($r = -0.31$, $P=0.03$) as well as TNFR1 and PPD \geq 5mm ($r=-0.32$, $P=0.003$). Results are presented in Figure 9. A further group-wise analysis was also done to observe the effects of PD on correlations between serum analytes and clinical parameters. The highest number of correlations were seen in the RA with PD group in which RANKL ($r= -0.46$), OPN ($r=-0.62$) and TNFR1 ($r = -0.51$) correlated negatively with BOP. Levels of RANKL ($r= -0.5$), OPN ($r = -0.57$) and TNFR1 ($r = -0.62$) also correlated negatively with number of shallow pockets (PPD 3-<5mm) in this group. In the PD group, an inverse correlation was seen for TNFR1 with MBL ($r = -0.45$).

Serum levels of OPN, RANKL, TNFR1, TNFR2, OPG and RANKL/OPG ratio

Comparison of serum concentrations between the four groups for OPN, RANKL, TNFR1, TNFR2 OPG levels and RANKL/ OPG ratio are shown in Table 8. RA with PD subjects had the highest medians for OPN, RANKL, TNFR2 and OPG. RA without PD subjects and the PD group had comparable levels for all four markers. RANKL levels were comparable between the RA (with and without PD) and PD groups. All markers were increased in RA subjects suffering from PD as compared to those without. There was no difference between the PD and RA without PD group. The analyses for RANKL/OPG ratio revealed that the ratios were comparable between PD and RA groups, however the ratio differed between the RA groups, with higher levels prevailing in those without PD. The PD and both RA groups had increased ratios in comparison to healthy subjects.

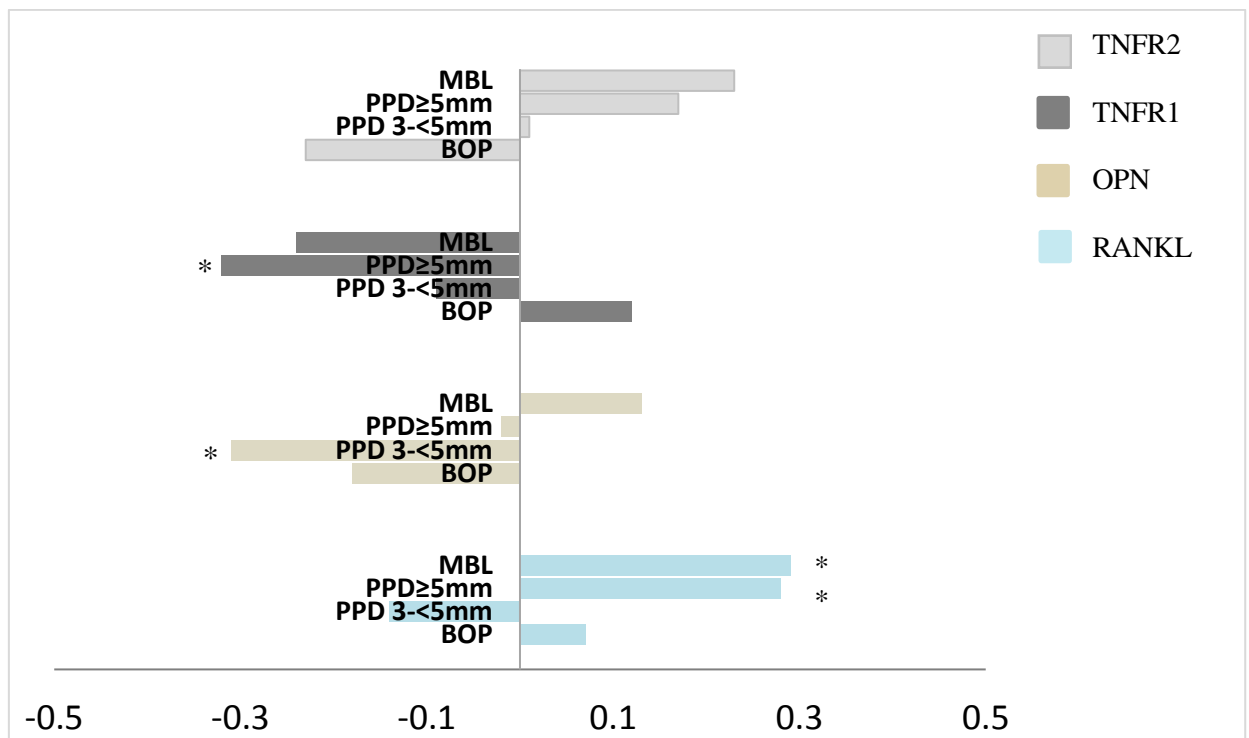


Figure 9. Correlations between clinical and serological data for all study subjects (n =90). The x axis represents Spearman r coefficient.

* Adjusted P-value < 0.05

Table 8. Serum levels of OPN, RANKL, TNFR1, TNFR2, OPG and RANKL/OPG ratio in RA (with and without PD), PD and healthy groups

	PD (n=38)	RA with PD (n=19)	RA without PD (n=19)	H (n=14)
OPN (pg/ml)	1332 ± 1135 ^{c f}	4021.7 ± 5393.5 ^b	1202.2 ± 874.6	3454 ± 1764.5 ^b
RANKL (pg/ml)	224 ± 117.2 ^f	242 ± 116 ^{b f}	183.5 ± 69 ^d	70 ± 113.5
TNFR1 (pg/m)	266.6 ± 618.2 ^{c e}	931.4 ± 1007 ^{b e}	141.2 ± 451	963.5 ± 745
TNFR2 (pg/ml)	4113 ± 1731.2 ^{c d}	4836 ± 3270.4 ^b _e	3938 ± 1975.5	3320 ± 2056
OPG (au)	10.06 ± 0.52 ^{c d}	10.34 ± 0.49 ^{b f}	10.15 ± 0.55	9.82 ± 0.25
RANKL/OPG (au)	0.77 ± 0.07 ^e	0.72 ± 0.07 ^{b d}	0.78 ± 0.06 ^e	0.64 ± 0.1

a compared to RA without PD. P<0.05

b compared to RA without PD. P<0.01

c compared RA with PD. P<0.05

d compared to healthy controls. P<0.05

e compared to healthy controls. P<0.01

f compared to healthy controls. P<0.001

All values are shown as median ± IQR

au = arbitrary units

Correlation between analytes

Pairwise correlation between analytes was done for all four study groups. The number of significant correlations was most frequently observed in the RA with PD group where RANKL correlated with OPN ($r = 0.55$) and TNFR1 ($r = 0.50$), OPN with TNFR1 ($r = 0.75$) and TNFR1 with TNFR2 ($r = 0.52$). In RA subjects without PD, TNFR1 correlated with RANKL ($r=0.63$), OPN ($r = 0.50$) and TNFR2 ($r = 0.49$). In PD subjects, TNFR1 correlated with OPN ($r = 0.46$) and TNFR2 ($r = 0.58$). TNFR1 and R2 also correlated significantly in the healthy group ($r = 0.54$).

STUDY IV

In this study, we measured PD severity using a continuous index of PPD Total and PPD Disease in subjects with and without rheumatoid arthritis. We also analysed glycated hemoglobin levels.

General characteristics for all study subjects

The total number of participating subjects was 118. Forty-seven subjects were with established RA disease. The total number of subjects suffering from periodontal disease was 77 (with RA = 26, without RA= 51). Healthy controls comprised of 20 persons.

Σ PPD Total, Tooth adjusted Σ PPD Total, MBL and HbA1c levels in study groups

Results for comparison between the four groups for the Σ PPD Total index and tooth adjusted Σ PPD Total are shown in Table 9. For both the indices, the medians for the PD group differed significantly from the remaining three groups as the group showed the highest values, followed by RA with PD and RA without PD. All groups differed from the healthy controls. There was no statistical difference between the RA groups for Σ PPD Total but after tooth adjustment, the difference was significant. For subjects suffering from PD (PD=51 and RA with PD = 26), Σ PPD Disease was more severe in subjects with PD only. Increased MBL was seen for the PD group with the highest median as compared to the remaining groups. MBL levels were comparable between RA without PD and healthy controls. The serum levels of HbA1c were the highest in PD group which differed significantly from RA with PD and healthy groups. However, HbA1c levels were comparable between PD and RA without PD subjects. Both the RA groups as well as PD had significantly higher HbA1c levels in comparison to healthy subjects.

Correlation of glycated hemoglobin with Σ PPD Total, Σ PPD Disease and MBL

Glycated hemoglobin levels were found to correlate with Σ PPD Total ($r = 0.36$, $P < 0.0001$) for all subjects ($n=118$). For subjects with PD ($n=77$), a correlation analysis between HbA1c and Σ PPD Disease also showed a significant correlation ($r = 0.25$, $P < 0.05$). A significant association was also found between glycated hemoglobin levels and MBL for all 118 subjects ($r = 0.25$, $P < 0.01$). The correlation was not significant between HbA1c and MBL in subjects with PD ($r = 0.21$, $P = 0.07$).

Correlation of Tooth adjusted Σ PPD Total with periodontal parameters

The correlation analyses between tooth adjusted Σ PPD Total and periodontal parameters showed a direct correlation with $PPD \geq 5\text{mm}$ ($r = 0.54$, $P < 0.000001$) and MBL ($r = 0.48$, $P < 0.0001$). BOP ($r = -0.26$, $P < 0.05$) and Σ PPD Disease ($r = -0.27$, $P < 0.05$) correlated showed an inverse relationship between numbers of teeth and inflammatory state. No correlation was seen for shallow pockets.

Table 9. Σ PPD Total, Tooth adjusted Σ PPD Total, MBL and HbA1c in RA (with and without PD), PD and healthy groups

	PD (n=51)	RA with PD (n=26)	RA without PD (n=21)	H (n=20)
Σ PPD Total / mm	384 \pm 99 ^{b e g}	301 \pm 73 ^g	276 \pm 74 ^g	191 \pm 21.5
Tooth adjusted Σ PPD Total	15.5 \pm 3.8 ^{b e g}	11.7 \pm 2.9 ^{a g}	10.8 \pm 2.5 ^g	6.8 \pm 0.9
Σ PPD Disease / mm	229 \pm 135 ^e	108 \pm 96	NA	NA
MBL / mm	5.24 \pm 2.01 ^{b c g}	4.57 \pm 0.9 ^{b g}	3.02 \pm 0.9	2.92 \pm 0.7
HbA1c (%)	5.7 \pm 1.2 ^{d g}	5.2 \pm 1 ^f	5.4 \pm 1.8 ^g	4.4 \pm 0.7

a compared to RA without PD. P<0.05
b compared to RA without PD. P<0.001
c compared to RA with PD. P < 0.05
d compared to RA with PD. P<0.01
e compared to RA with PD. P<0.001
f compared to healthy controls. P<0.05
g compared to healthy controls. P<0.001
 All values are shown as median \pm IQR
 NA = not applicable

Correlation of Σ PPD Disease and Σ PPD Total with number of deep pockets and MBL

To assess the sensitivity of Σ PPD disease, a correlation analyses was done with number of PPD \geq 5mm and MBL for subjects suffering from PD (n = 77). The results showed a strong correlation with PPD \geq 5mm (r = 0.83, P<0.0001) whereas for MBL the correlation was (r = 0.28, P<0.05). Σ PPD Total correlated strongly as well with PPD \geq 5mm (r = 0.72, P < 0.0001) but not with MBL (r = 0.13, P = 0.25).

DAS 28 correlation with HbA1c, rheumatological and periodontal parameters in RA subjects with and without PD

In order to assess the degree of correlation between RA disease activity using DAS28 and parameters of rheumatologic (IgM-RF, ESR) and periodontal interest (BOP, Σ PPD Total, Σ PPD Disease, MBL PPD 3-<5mm, PPD \geq 5mm) as well as glycated hemoglobin levels and age, correlation analyses was carried out. The results showed only three significant correlations. An inverse correlation was found between DAS28 and PPD 3-<5mm (r = -0.68, P<0.001) in RA with PD subjects. DAS28 was directly associated with IgM-RF levels in both the RA groups with PD (r = 0.39, P<0.05) and without PD (r = 0.47, P < 0.05).

DISCUSSION

STUDY I

The results from *study I* show that low LRP1 expression on T lymphocytes is due to continuous shedding (Figure 10). This LRP1 shedding is spontaneous in nature rather than induced by exogenous stimuli which makes it unique (Gorovoy et al. 2010). T cells are known to express ADAM10 (Ebsen et al., 2013).

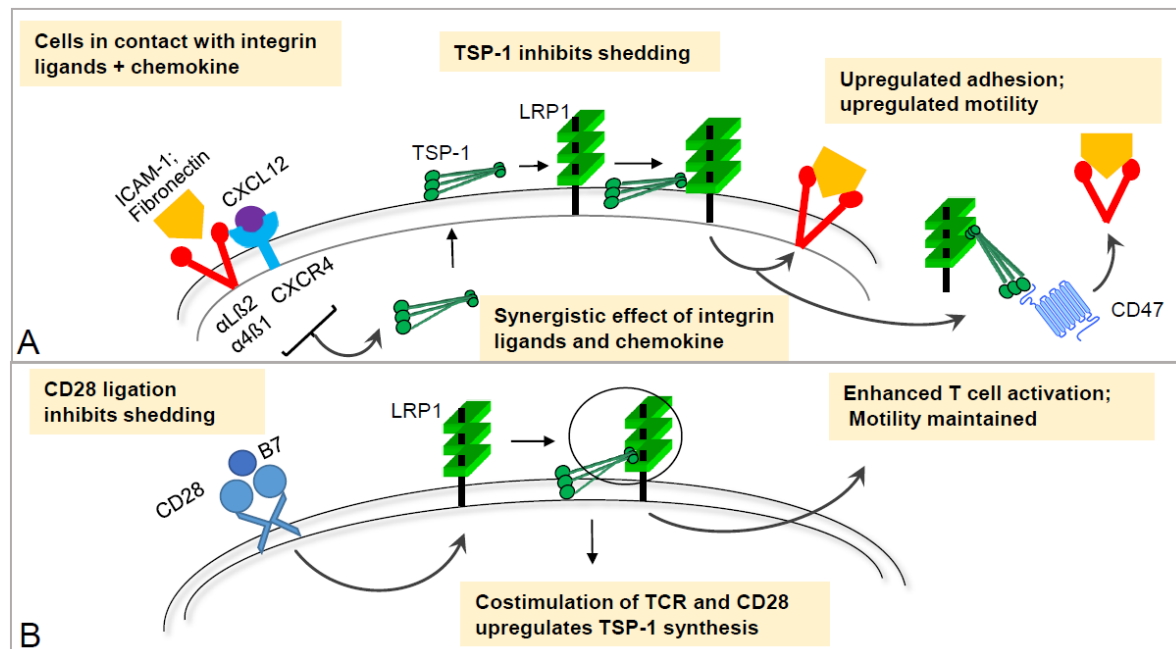


Figure 10. Mechanisms underlying T cell regulation through LRP1. (A) T cell membrane receptors in contact with integrin ligands and CXCL12 induces cell surface expression of TSP-1 which in turn inhibits shedding of LRP1. (B) CD28 ligation inhibits shedding of LRP1.

Using a broad spectrum metalloprotease (MMP) inhibitor GM6001, we were able to demonstrate the profound effect of this protease inhibitor on LRP1 expression on T cells. This indicates that LRP1 is transported to the cell surface and then released by an enzymatic mechanism. TSP-1 is one of the many ligands that binds to LRP1. We showed in our study that TSP-1 associates heavily with LRP1 once TSP-1 transport to the cell surface has been stimulated by cell contact with $\beta 1$ and $\beta 2$ integrin ligands.

The preformed TSP fragments TSP170 and TSP130 may be responsible for inhibiting the sheddase, thus upregulating LRP1. TSP130 was exclusively expressed in adherent cells indicating its pro-adhesive property. Our results suggest that preformed TSP fragments via association with LRP1 determine the behavior of T cells with respect to adhesive interaction with extracellular matrix, antigen-presenting cells or endothelial cells.

For efficient T-cell activation, both the recognition of antigen by TCR as well as engagement of costimulatory molecules are vital. CD28 is widely recognized as the major costimulatory protein for naive T-cell activation, and the CD28/B7 pathway plays a central role in immune responses (Bour-Jordan et al., 2002). Our findings show that CD28 ligation also inhibits shedding of LRP1, although independent of TSP-1. Hence, it is very likely that co-stimulation may upregulate cell signaling through upregulated T cell expression of LRP1 and TSP-1. The LRP1 shedding is a regulatory mechanism directed towards immunosuppression and the antagonistic co-stimulatory pathways may have evolved to combine defense against pathogens with protection against excessive and adverse immune responses.

STUDY II

In *study II* we analysed 92 human proteins related to immuno-inflammatory processes in RA and PD subjects. We found significant correlations between several proteins and periodontal parameters. Our findings reported the association for three novel markers with disease severity consistently. FGF-19, ST1A1 and NT3 correlated positively whereas EN-RAGE, DNER, TWEAK and CX3CL1 correlated negatively with BOP, PPD \geq 5mm and MBL. There are no previous studies on the association of FGF-19, ST1A1 and NT3 with either PD or RA. EN-RAGE, DNER, TWEAK and CX3CL1 are proteins abundantly expressed by leucocytes as well as neuronal cells (DNER) which, collectively, are either recruiters or stimulators of other key mediators of inflammation. The negative correlation between the serological levels of these markers and periodontal parameters may be due to their increased engagement and/or consumption at tissue receptor sites undergoing active inflammation.

We also found significant correlation between antigen specific receptor complex associated proteins CD5 and CD6. They are found on TCR and BCR and participate in the modulation of signals that allow activation and /or differentiation of T or B cells, depending on the signals received from the antigen-receptor complex. A recent study by Orta-Mascaró et al. found that CD6 modulates the threshold for thymocyte selection of T cells as well as the generation and/or function of several peripheral T cell subpopulations (Orta-Mascaró et al., 2016). Low levels of CD5 have been found in the serum of healthy individuals, with increasing levels reported in some autoimmune diseases such as RA (Jamin et al., 1992) and primary Sjögren's syndrome (Ramos-Casals et al., 2001). Soluble CD5 has been shown to function as a negative regulator of T and B cell responses resulting in enhanced activation of effector T and B cells. The direct association of CD5 and CD6 levels with periodontal parameters indicates an anomalous T cell response in PD.

Further analysis also revealed an inverse correlation for both IL-10 and its receptors with periodontal parameters (PPD 3-<5mm, MBL). This was also observed in RA with PD subjects (BOP, PPD 3-<5mm and \geq 5mm). This inverse correlation mirrors a compromised regulatory mechanism which allows to sustain inflammation. IL-10 is anti-inflammatory known to potently inhibit TNF- α production along with other pro-inflammatory cytokines, including IL-1 and IL-6 (Moore et al., 2001, Brennan et al., 2008). For the exception of CCL25, most chemokines associated inversely with clinical parameters. This may be explained in part by the RA group receiving strong anti-inflammatory medication (NSAIDs, corticosteroids, DMARDs) which can exert an inhibitory effect on chemokine production (Szekanecz et al., 2010).

Apoptosis associated proteins were also found to be associated with disease severity. CASP-8 with BOP and PPD \geq 5mm, HGF with PPD \geq 5mm, and CDCP1 with MBL indicate that inflammatory and apoptotic processes may be interlinked in PD. There is evidence for the role of CASP-8 in leading osteoblasts to early programmed cell death via prior sensitization to apoptotic signals (Mori et al, 2009). HGF levels in GCF and saliva have been shown to increase proportionally with the severity of PD (Nagaraja & Pradeep, 2007). HGF is also an osteoclast secreted clastokine with a role in inducing osteopontin production by osteoblasts (Chen et al., 2012).

STUDY III

In *study III*, we investigated concentrations of OPN, RANKL, TNFR1, TNFR2, OPG and the RANKL/OPG ratio in serum samples from subjects suffering from periodontal disease alone, suffering from rheumatoid arthritis with and without periodontal disease and healthy groups.

Osteopontin (OPN) is a phosphoprotein mainly secreted extracellularly. It can regulate mineral crystal growth as well as mediate cell attachment and signaling via recognition of integrins (Sodek et al., 2013). OPN is recognized as a matricellular protein as well as a cytokine involved in the recruitment of immune cells in inflammation (Merry et al., 1993, Rittling et al., 1998). Our results showed differences in OPN levels between the PD and RA subjects suffering from PD. This may be explained by the fact that soluble OPN is released by both osteoblasts and osteoclasts which may negatively regulate osteoclast precursor formation as part of a negative feedback mechanism (Giachelli et al., 2000). This mechanism may be a compensatory response in an attempt to maintain bone levels in osteolytic diseases, as reflected by higher serum levels of OPN seen in RA with PD subjects.

Serum levels of RANKL in PD subjects were comparable to both the RA groups which shows an extensive involvement of immune cells in PD. This is a highly notable finding given that osteolysis in RA involves multiple sites as compared to alveolar bone loss of the jaws in PD. Higher RANKL levels were also associated positively with two clinical parameters of disease severity, PPD ≥ 5 mm and MBL. This finding supports that of a recent study in which RANKL serum levels were also associated with PPD (Baltacıoğlu et al., 2014). Besides serum, RANKL concentration levels in periodontal tissues have been shown to correlate with increasing pocket depth (Kawai et al., 2006). Elevation of RANKL has also been observed in gingival crevicular fluid (GCF) in PD patients (Bostanci et al., 2007). The cellular sources of RANKL include osteoblasts, dendritic cells, periodontal ligament fibroblasts but mostly B and T cells (Bertl et al., 2012). For bone destruction to take place, a certain critical level of RANKL is required for immuno-inflammatory signals to orchestrate bone loss (Lam et al., 2000).

RANKL exists in both membrane-bound form and as a soluble form in circulation. In the circulation, it also exists in a bound form, complexed with OPG. OPG itself circulates in different forms, as a monomer, a dimer, and as a RANKL/OPG conjugate (Rogers et al., 2005). Therefore, calculation of the ratio of RANKL to OPG was a relevant marker which was found to be increased in both PD and RA groups as compared to healthy subjects. Based on the fact that high RANKL/OPG ratio influences osteoclast activity, comparable levels of this ratio between RA and PD groups indicate an overall systemic bone resorptive status. Soluble TNF receptors has been suggested as negative modulators of TNF activity, therefore under normal conditions, TNF receptor concentrations exceed those of TNF. TNFR1 and TNFR2 both inhibit TNF- α , although binding to TNFR1 (177-fold) as compared to that of TNFR2 occurs with more ardency (Terlizzese et al., 1996). In this study, an inverse correlation was found between TNFR1 levels and both MBL and deep pockets (≥ 5 mm) in the PD group. Previous experimental studies have highlighted the antagonizing effects of soluble receptors on TNF- α , thereby preventing TNF-mediated bone loss (Assuma et al., 1998). Deep periodontal pockets form as a result of periodontal tissue attachment loss which is mediated by strong pro-inflammatory cytokines such as TNF- α . In the absence of

physiological TNF inhibitors, TNF mediated tissue loss will persist (Delima et al., 2001). TNFR1 concentrations tend to be higher in the sera of RA patients (Cope et al., 1992). Our findings also showed TNFR1 levels to be the highest in the RA with PD group. This may reflect the accumulation of TNFR1 shedding in inflamed periodontal tissues as well as synovial inflammation. TNF receptors have also been detected in GCF where PPD values were higher (Ikezawa et al., 2005). The level of TNFR2 were also the highest in the RA with PD group, decreasing across remaining groups. High TNFR2 levels in RA patients have been reported before (Barton et al., 2001).

The differences in shedding mechanisms may explain the difference between TNFR1 and TNFR2 levels. TNFR1 is pro-apoptotic while TNFR2 signals cell survival (Faustman et al., 2013). In autoimmune diseases, defects such as polymorphisms in the TNFR2 gene may up regulate its expression and shedding. This excessive shedding may be a mechanism for indirectly antagonizing TNFR1 which would consequently reduce TNF- α activity. Since previous studies have also shown levels of soluble TNFR2 exceeding those of TNFR1, this emphasizes the dominance of TNFR2 in order to be able to down-regulate TNF- α responses (Carpenter et al., 1995).

Determination of the level of co-regulation between the osteoclastogenic markers showed that TNFR1 and TNFR2 were significantly associated in all four groups, with the strongest coefficient in the PD group. TNFR1 correlated with all three markers (OPN, RANKL and TNFR2) in both RA groups. For the PD group, TNFR1 was found to correlate with OPN but not with RANKL. TNF α is an inhibitor of bone formation as it stimulates, via TNFR1, the production of Dickkopf-1 (DKK-1). DKK-1 is a secreted glycoprotein which inhibits a cell signaling pathway that controls tissue regeneration in adult bone marrow (Diarra et al., 2007). Apoptosis of periodontal ligament cells in PD via TNFR1 stimulation is another mechanism which reduces bone formation as this diminishes the number of osteoblast precursor cells (Graves et al., 2011).

STUDY IV

In *study IV*, we examined periodontal disease severity using a new index Σ PPD Total and Σ PPD disease and examined the glycemic regulatory status by measuring HbA1c levels in subjects with and without RA. As knowledge regarding the etiopathogenesis of PD increases with research, so does the need to discover and employ improved methods of disease measurement.

The selection of a disease measuring index should be composed of assessments which are governed by parameters sensitive to change. Periodontal pocket formation is one such parameter considered in terms of tissue responsiveness to inflammation. Inflammatory changes cause the fibroblasts and collagen fibers around the epithelial ridges to be lost only to be replaced by immuno-inflammatory cells. Epithelial attachment at the base of the pocket extends not only apically but horizontally as well (Bosshardt, 2017). The response observed upon reduction of the inflammation is formation of a new epithelial attachment, thus reducing probing depth measurement (Saminsky et al., 2015). The rationale behind the use of continuous variables that are dichotomized in most periodontal research is that periodontal pocketing and bone loss do not occur uniformly throughout the entire dentition, so dichotomy helps overcome this uneven characteristic (Dye et al., 2002).

The main disadvantage of dichotomising is loss of information hence reducing the statistical power to detect a relation between the variable and outcome. Secondly and naturally, with loss of data, there is underestimation of variability between groups (Cohen, 1983). We, therefore, developed a continuous disease measurement index with three parts unified by the basic principle of recording a direct representation of the extent of PPD with no loss of data. The first part of the index, Σ PPD Total, is simply an arithmetic sum of all measurable sites per subject. The second part, Σ PPD Disease, is a sub-aggregate which provides the total sum of diseased sites. The final part is an adjustment of Σ PPD Total to the number of teeth. Therefore, tooth and site-specific information is included ensuring no loss of data. We performed a correlation analysis to determine the potential of the Σ PPD Total index validity. Our results showed a high degree of correlation between all three of its components and number of deep pockets. The inverse correlation between tooth adjusted ratio and PPD disease was indicative of disease severity as higher Σ PPD disease correlated with lesser number of teeth and vice versa. Furthermore, our results revealed that the tooth adjusted Σ PPD total was able to gauge disease severity adequately as it associated significantly with parameters of PD severity.

The disease activity measurement for RA employed the DAS28 index which is a composite continuous index comprising of several component variables, including swollen and tender joint count, with a high contribution of acute-phase reactant (CRP or ESR) levels.

Radiographic progression in RA is known to be associated with swollen joint count and acute-phase reactants. DAS28, based on ESR, correlated with IgM-RF values in both RA with and without PD subjects which has also been reported by another study (Ursum et al., 2010). Auto-antibody RF is one of the most powerful predictors of joint damage in early RA and RF positive RA patients demonstrate an increase in the DAS28 score in relation to an increase in radiologic damage (Welsing et al., 2004). Interestingly, this association did not seem to be affected by the status of periodontal health amongst RA subjects. Perhaps due to its composite nature and complex pathogenesis of RA, little or no association has been reported between DAS28 and periodontal parameters. However, we found that the number of

shallow pockets was the only periodontal parameter to be significantly and inversely correlated with DAS28 in RA with PD subjects. This may partly be explained by the fact that serum inflammatory cytokines and chemokines were also found to correlate inversely with shallow pockets in RA subjects with PD, aligning the serological representation with clinical activity (Panzai et al., 2017).

When it comes to a glycemic disorders or metabolic disease such as diabetes mellitus, laboratory measures reflect severity. The persistence of inflammatory cytokines can play a crucial role in the development of insulin resistance both in RA and PD patients, placing them at risk for T2D (Solomon et al., 2010, Gaurav, 2012). In order to assess and compare the extent of glycemic disorder between RA and PD subjects, HbA1c levels were investigated. Our results showed a direct association between HbA1c levels and PD severity in terms of PPD index and bone loss. These findings are similar to that of a recent study where HbA1c levels were found to be higher amongst subjects with severe periodontal disease (Teeuw et al., 2017).

Serum levels of HbA1c showed that glycemic disorder was the greatest in PD group, although statistically, there was no difference on comparison with the RA without PD group. We also found in *study III* their osteoimmunological inflammatory profile to be comparable (Panzai et al., 2018). Although all three disease groups showed higher HbA1c levels as compared to healthy controls, the lower levels in RA subjects in comparison to PD maybe explained by the use of disease modifying anti-rheumatic drugs (DMARDs) in this group. Certain DMARDs such as methotrexate and hydroxychloroquine have been shown to improve insulin sensitivity which reduces HbA1c levels in RA subjects (Rekedal et al., 2010).

CONCLUSIONS

This thesis shows that periodontal disease is mirrored by a range of systemic immune markers, particularly those involved in inflammation. Furthermore, peripheral osteoclastogenesis is a feature of PD, comparable to RA. In addition, it also signifies the peripheral involvement of host immune system and its effects on the glycemic regulation in both PD and RA groups. The use of a simple summation of several variables without dichotomising provides the basis for sensitive approach towards PD measurement. The thesis also shows evidence that LRP1 controls motility, adhesion and activation in T cells.

FUTURE PERSPECTIVES IN LIGHT OF NOVEL FINDINGS

Periodontal conditions are diagnosed clinically and radiographically however their severity is not limited to the confines of the oral cavity. PD is well reflected by host derived systemic inflammatory cytokines, chemokines, enzymes and growth factors.

Peripheral Osteoclastogenesis and PD

This thesis showed that serum levels of osteoclastogenic marker RANKL and bone resorptive status (RANKL/OPG ratio) was comparable between PD and RA disease groups. In light of this, further research will focus on gauging PD osteolytic effects in comparison to subjects with cardiovascular diseases (CVD) and T2D.

Associated biomarkers and PD

Due to high inter individual variability, correlation statistical analyses was chosen over inter group comparison when measuring an extensive array of biomarkers (*study II*) which revealed a clear consistency for novel markers FGF-19, ST1A1 and NT3 directly associated with PD parameters (BOP, PPD \geq 5mm and MBL). Further research will be planned capitalizing on these findings and studying these markers in subjects with PD and attendant comorbidities such as CVD and T2D.

PPD Total and PPD Disease index

Further research regarding the use of a novel index, Σ PPD Total and Σ PPD Disease are required to validate its use and establish its sensitivity and specificity in comparison to other indices.

POPULAR SCIENCE

Periodontal disease (PD) affects the supporting tissues around the tooth including gum and bone. It is the leading cause of tooth loss in adults. The bacteria in oral plaque has been attributed as a trigger for periodontal disease, however there is enough supporting evidence to show that it is the immune response of the host that is responsible for the severity of the disease.

This thesis focused on elucidating the involvement of the host response in subjects who were suffering from PD. They were compared with subjects suffering from rheumatoid arthritis (RA), an autoimmune disease which involves a strong systemic immune response in a self-directed attack against synovial joints. With this comparison, it was possible to gauge the extent of the immune response involvement in combating PD as an inflammatory disease.

In *study II*, we measured the levels of 92 protein markers, most of which are known to play a role in inflammation, and performed analyses which would allow us to determine whether these markers were associated with periodontal conditions or not. In *study III*, we measured another set of markers which are specific for bone resorption. Again, we performed analyses to assess any association between PD severity and markers of bone resorption. In *study IV*, we developed a new method of measuring PD clinically and tried to elucidate any correlation with glycated hemoglobin blood levels.

Our results showed that PD inflammation is indeed well reflected by markers of inflammation measurable in the blood. Most importantly, the levels for markers of bone resorption were similar between RA and PD groups. This is an important finding which shows the extent of inflammation imparted by poor periodontal health.

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REFERENCES

- Abusleme L, Dupuy AK, Dutzan N, Silva N, Burleson JA, Strausbaugh LD, Gamonal J, Diaz PI. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J.* 2013; 7:1016–1025.
- Aggarwal R, Ringold S, Khanna D, Neogi T, Johnson SR, Miller A, Brunner HI, Ogawa R, Felson D, Ogdie A, Aletaha D. Distinctions between Diagnostic and Classification Criteria? *Arthritis care & research.* 2015; 67:891-897.
- Albandar JM, Brunelle JA, Kingman A. Destructive periodontal disease in adults 30 years of age and older in the United States, 1988–1994. *J Periodontol.* 1999; 70:13–29.
- Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham III CO, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B. Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Ann Rheum Dis.* 2010; 69: 1580–1588.
- Allam JP, Duan Y, Heinemann F, Winter J, Gotz W, Deschner J, Wenghoefer M. IL-23 producing CD68 macrophage-like cells predominate within an IL-17-polarized infiltrate in chronic periodontitis lesions. *J Clin Periodontol.* 2011; 38: 879-886.
- Altamash M, Arledal S, Klinge B, Engström PE. Pre-diabetes and diabetes: Medical risk factors and periodontal conditions. *Acta Odontol Scand.* 2013; 71:1625-1631.
- Altamash M, Klinge B, Engström PE. Periodontal treatment and HbA1c levels in subjects with diabetes mellitus. *J Oral Rehabil.* 2016; 43:31-38.
- American Academy of Periodontology Task Force Report on the Update to the 1999 Classification of Periodontal Diseases and Conditions. *J Periodontol.* 2015; 86: 835–838.
- Anderson SG, Bentzon MW, Houba V, Krag P. International reference preparation of rheumatoid arthritis serum. *Bull WHO.* 1970; 42:311–8.
- Andersson A, Stubelius A, Karlsson MN, Engdahl C, Erlandsson M, Grahne L, Lagerquist MK, Islander U. Estrogen regulates T helper 17 phenotype and localization in experimental autoimmune arthritis. *Arthritis Res Ther.* 2015; 17:32.
- Angelotti F, Parma A, Cafaro G, Capecchi R, Alunno A, Puxeddu I. One year in review. Pathogenesis of RA. *Clin Exp Rheumatol.* 2017; 35: 368-378.
- Bahrami G, Væth M, Isidor F, Wenzel A, Vaeth M. Marginal bone loss over 5 years in an adult Danish population. *Oral Health Prev Dent.* 2007; 5:113-118.
- Balandraud N, Meynard JB, Auger I, Sovran H, Mugnier B, Revirion D, Roudier J, Roudier C. Epstein-Barr virus load in the peripheral blood of patients with rheumatoid arthritis: accurate quantification using real-time polymerase chain reaction. *Arthritis Rheum.* 2003; 48:1223-1228.
- Baltacıoğlu E, Kehribar MA, Yuva P, Alver A, Atagün ÖS, Karabulut E, Akalın FA. Total oxidant status and bone resorption biomarkers in serum and gingival crevicular fluid of patients with periodontitis. *J Periodontol.* 2014; 85: 317-326.

- Bergström J, Preber H. Tobacco use as a risk factor. *J Periodontol*. 1994; 65:545-50.
- Bertl K, Pietschmann P, Matejka M. Osteoimmunological Aspects of Periodontal Diseases (ed. Pietschmann P) *Principles of Osteoimmunology*. Vienna: Springer; 2012; 241-273.
- Borrell LN, Crawford ND. Socioeconomic position indicators and periodontitis: Examining the evidence. *Periodontol 2000*. 2012; 58:69-83.
- Bosshardt DD. The periodontal pocket: pathogenesis, histopathology and consequences. *Periodontol 2000*. 2018; 76: 43-50.
- Bostanci N, Belibasakis GN. *Porphyromonas gingivalis*: an invasive and evasive opportunistic oral pathogen. *FEMS Microbiol Lett*. 2012; 333:1-9.
- Bostanci N, İlgenli T, Emingil G, Afacan B, Han B, Töz H, Atilla G, Hughes FJ, Belibasakis GN. Gingival crevicular fluid levels of RANKL and OPG in periodontal diseases: implications of their relative ratio. *J Clin Periodontol*. 2007; 34:370-376.
- Bour-Jordan H, Bluestone JA. CD28 function: a balance of costimulatory and regulatory signals. *J Clin Immunol*. 2002; 22:1-7.
- Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature*. 2003; 423:337–342.
- Brennan FM, Green P, Amjadi P, Robertshaw HJ, Alvarez-Iglesias M, Takata M. Interleukin-10 regulates TNF- α -converting enzyme (TACE/ADAM-17) involving a TIMP-3 dependent and independent mechanism. *Eur J Immunol*. 2008; 38: 1106–1117.
- Chakravarti A, Raquil MA, Tessier P, Poubelle PE. Surface RANKL of Toll-like receptor 4-stimulated human neutrophils activates osteoclastic bone resorption. *Blood*. 2009; 114:1633-1644.
- Chen HT, Tsou HK, Chang CH, Tang CH. Hepatocyte growth factor increases osteopontin expression in human osteoblasts through PI3K, Akt, c-Src, and AP-1 signaling pathway. *PLoS One*. 2012; 7:e38378.
- Ciccarelli F, Martinis MD, Ginaldi L. An Update on Autoinflammatory Diseases. *Curr Med Chem*. 2014; 21:261-269.
- Crowson CS, Matteson EL, Myasoedova E, Michet CJ, Ernste FC, Warrington KJ, Davis III JM, Hunder GG, Thorneau TM, Gabriel SE. The lifetime risk of adult-onset rheumatoid arthritis and other inflammatory-autoimmune rheumatic diseases. *Arthritis Rheum*. 2011;63; 633 639.
- del Rincón I, Battafarano DF, Arroyo RA, Murphy FT, Fischbach M, Escalante A. Ethnic variation in the clinical manifestations of rheumatoid arthritis: role of HLA–DRB1 alleles. *Arthritis Care Res*. 2003; 49:200-208.
- Dembic, Z. The cytokines of the immune system the role of cytokines in disease related to immune response. Burlington :: Elsevier Science. 2015.
- Díaz-Zúñiga J, Melgar-Rodríguez S, Rojas L, Alvarez C, Monasterio G, Carvajal P, Vernal R. Increased levels of the T-helper 22-associated cytokine (interleukin-22) and transcription factor (aryl hydrocarbon receptor) in patients with periodontitis are associated with osteoclast resorptive activity and severity of the disease. *J Periodont Res*. 2017; 52:893.

- Donati M, Liljenberg B, Zitzmann NU, Berglundh T. B-1a cells and plasma cells in periodontitis lesions. *J Periodont Res*. 2009; 44:683-688.
- Doria A, Zen M, Bettio S, Gatto M, Bassi N, Nalotto L, Ghirardello A, Iaccarino L, Punzi L. Autoinflammation and autoimmunity: bridging the divide. *Autoimmun Rev*. 2012; 12:22-30.
- Dye BA, Choudhary K, Shea S, Papapanou PN. Serum antibodies to periodontal pathogens and markers of systemic inflammation. *J Clin Periodontol*. 2005; 32: 1189–1199.
- Ebsen H, Schröder A, Kabelitz D, Janssen O. Differential surface expression of ADAM10 and ADAM17 on human T lymphocytes and tumor cells. *PloS One*. 2013; 8:e76853.
- Eke PI, Dye BA, Wei L, Thornton-Evans GO, Genco RJ. Prevalence of periodontitis in adults in the United States: 2009 and 2010. *J Dent Res* 2012; 91: 914–920.
- Figueira EA, de Rezende ML, Torres SA, Garlet GP, Lara VS, Santos CF, Avila-Campos MJ, da Silva JS, Campanelli AP. Inhibitory signals mediated by programmed death-1 are involved with T cell function in chronic periodontitis. *J Periodontol*. 2009; 80:1833-44.
- Forslind K, Ahlmén M, Eberhardt K, Hafström I, Svensson B. Prediction of radiological outcome in early rheumatoid arthritis in clinical practice: role of antibodies to citrullinated peptides (anti-CCP). *Ann Rheum Dis*. 2004; 63: 1090–1095.
- Garlet PG, Aranha MF, Silveira ME, Vieira AE, Queiroz-Junior CM, Madeira M, Fukada SY, Tarcilia A, Silva TA. The role of chemokines and cytokines in the pathogenesis of periodontal and periapical lesions: Current concepts. In: Khatami M, *ed*. *Inflammation, Chronic Diseases and Cancer: Cell and Molecular Biology, Immunology and Clinical Bases*. Rijeka, Croatia: InTech; 2012: 219–264.
- Giachelli CM, Steitz S. Osteopontin: a versatile regulator of inflammation and bio mineralization. *Matrix Biol*. 2000; 19: 615-22.
- Gorovoy M, Gaultier A, Campana WM, Firestein GS, Gonias SL. Inflammatory mediators promote production of shed LRP1/CD91, which regulates cell signaling and cytokine expression by macrophages. *J Leukoc Biol*. 2010; 88: 769-778.
- Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum*. 1987; 30: 1205–1213.
- Grossi SG, Genco RJ, Machtei EE, Ho AW, Koch G, Dunford R, Zambon JJ, Hausmann E. Assessment of risk for periodontal disease. II. Risk indicators for alveolar bone loss. *J Periodontol*. 1995; 66: 23–29.
- Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG, Machtei EE, Norderyd OM, Genco RJ. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *J Periodontol*. 1994; 65: 260–267.
- Gurav AN. Periodontitis and Insulin Resistance: Casual or Causal Relationship? *Diabetes & Metabolism Journal*. 2012; 36:404-411.
- Gurung P, Burton A, Kanneganti TD. NLRP3 inflammasome plays a redundant role with caspase 8 to promote IL-1 β -mediated osteomyelitis. *Proceedings of the National Academy of Sciences*. 2016; 113:4452-7.

Hajishengalis G a. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts and host response. *Trends Immunol.* 2014; 35:3-11.

Hajishengallis G b. The inflammophilic character of the periodontitis-associated microbiota. *Mol Oral Microbiol.* 2014; 29: 248–257.

Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskandari MA, McIntosh ML, Alsam A, Kirkwood KL, Lambris JD, Darveau RP. Low abundance biofilm species orchestrates inflammatory periodontal disease through commensal microbiota and complement. *Cell Host Microbe.* 2011; 10: 497-506.

Hameed K, Gibson T. A comparison of the prevalence of rheumatoid arthritis and other rheumatic diseases amongst Pakistanis living in England and Pakistan. *Br J Rheum.* 1997; 36: 781-785.

Hammond ME, Lapointe GR, Feucht PH, Hilt S, Gallegos CA, Gordon CA, Giedlin MA, Mullenbach G, Tekamp-Olson P. IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors. *J Immunol.* 1995; 155:1428-1433.

Hansen BF, Gjermo P, Bergwitz-Larsen KR. Periodontal bone loss in 15-year old Norwegians. *J Clin Periodontol.* 1984; 11:125–131.

Hausmann E, Allen K, Clerehugh V. What alveolar crest level on a bite-wing radiograph represents bone loss? *J Periodontol.* 1991; 62:570–572.

Hiemstra PS. Novel roles of protease inhibitors in infection and inflammation. *Biochem Soc Trans.* 2002; 30: 116-120.

Higgs BW, Zhu W, Richman L, Fiorentino DF, Greenberg SA, Jallal B, Yao Y. Identification of activated cytokine pathways in the blood of systemic lupus erythematosus, myositis, rheumatoid arthritis, and scleroderma patients. *Int J Rheum Dis.* 2012; 15: 25-35.

Hirschmann PN. The radiographic interpretation of periodontal disease. In: Derrick DD, editor. *The 1989 dental annual*, 1. London: Wright, 1989:170–81.

Hobdell MH, Oliveira ER, Bautista R, Myburgh NG, Lalloo R, Narendran S, Johnson NW. Oral diseases and socio-economic status (SES). *Br Dent J.* 2003; 194:91-6.

Hodge PJ, Robertson D, Paterson K, Smith GLF, Creanor S, Sherriff A. Periodontitis in non-smoking type 1 diabetic adults: a cross-sectional study. *J Clin Periodontol.* 2012; 39: 20–29.

Hoffman HM, Broderick L. The role of the inflammasome in patients with autoinflammatory diseases. *J Allergy Clin Immunol.* 2016; 138:3-14.

Ivanova EA, Orekhov AN. T Helper Lymphocyte Subsets and Plasticity in Autoimmunity and Cancer: An Overview. *BioMed Res Int.* 2015; 327470:1-9.

Hou JM, Lin JL, Wen JP, Jin L, Tang FQ. Immunohistochemical identification of osteoclasts and multinucleated macrophages. *Cell Immunol.* 2014; 292:53-56.

Jin L, Wong KY, Leung WK, Corbet EF. Comparison of treatment response patterns following scaling and root planing in smokers and non-smokers with untreated adult periodontitis. *J Clin Dent.* 2000; 11: 35–41.

- Johnston CJ, Smyth DJ, Dresser DW, Maizels RM. TGF- β in tolerance, development and regulation of immunity. *Cell Immunol.* 2016; 299:14-22.
- Källberg H, Ding B, Padyukov L, Bengtsson C, Rönnelid J, Klareskog L, Alfredsson L, EIRA Study Group. Smoking is a major preventable risk factor for rheumatoid arthritis: estimations of risks after various exposures to cigarette smoke. *Ann Rheum Dis.* 2011; 70, 508–511.
- Kassebaum NJ, Smith AG, Bernabé E, Fleming TD, Reynolds AE, Vos T, Murray CJ, Marcenes W, GBD 2015 Oral Health Collaborators. Global, regional, and national prevalence, incidence, and disability-adjusted life years for oral conditions for 195 countries, 1990–2015: a systematic analysis for the global burden of diseases, injuries, and risk factors. *J Dent Res.* 2017; 96:380-387.
- Kawai T, Matsuyama T, Hosokawa Y, Makihiro S, Seki M, Karimbux NY, Goncalves RB, Valverde P, Dibart S, Li YP, Miranda LA. B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. *Am J Pathol.* 2006; 169:987-998.
- Khan AA, Shareef I, Ayma S, Ambreena Q, Inayatullah P, Sofia S. Oral health in Pakistan: a situation analysis. *Dev Dent.* 2004; 5:35-44.
- Kinane DF, Lindhe J. Pathogenesis of periodontal disease. In: Lindhe J, ed. *Textbook of periodontology*. Copenhagen: Munksgaard, 1997.
- Kinane DF, Mooney J, Ebersole JL. Humoral immune response to *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in periodontal disease. *Periodontol* 2000. 1999; 20: 289-340.
- Kitani A, Fuss I, Nakamura K, Kumaki F, Usui T, Strober W. Transforming growth factor (TGF)- β 1-producing regulatory T cells induce Smad-mediated interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGF- β 1-mediated fibrosis. *J Exp Med.* 2003; 198:1179-88.
- Koga T, Inui M, Inoue K, Kim S, Suematsu A, Kobayashi E, Iwata T, Ohnishi H, Matozaki T, Kodama T, Taniguchi T. Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. *Nature.* 2004; 428:758-763.
- Krishnamurthy A, Joshua V, Hensvold AH, Jin T, Sun M, Vivar N, Ytterberg AJ, Engström M, Fernandes-Cerqueira C, Amara K, Magnusson M. Identification of a novel chemokine-dependent molecular mechanism underlying rheumatoid arthritis-associated autoantibody-mediated bone loss. *Ann Rheum Dis.* 2016; 75:721-729.
- Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell.* 1998 ; 93:165-176.
- Laine ML, Loos BG, Crielaard W. Gene polymorphisms in chronic periodontitis. *Int J Dent.* 2010; 324719: 1-22.
- Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL. TNF- α induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J Clin Invest.* 2000; 106: 1481–1488.

- Lang NP, Bartold PM. Periodontal health. *J Clin Periodontol*. 2018; 45(Suppl 20):S230–S236.
- Last JM, Abramson JH, Freidman GD, editors. *A dictionary of epidemiology*. New York: Oxford University Press; 2001.
- Lee DE, Kim JH, Choi SH, Cha JH, Bak EJ, Yoo YJ. Periodontitis mainly increases osteoclast formation via enhancing the differentiation of quiescent osteoclast precursors into osteoclasts. *J Periodont Res*. 2015; 50: 256–264.
- Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK. LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. *Physiol Rev*. 2008; 88:887-918.
- Liu Y, Aryee MJ, Padyukov L, Fallin MD, Hesselberg E, Runarsson A, Reinius L, Acevedo N, Taub M, Ronninger M, Shchetynsky K. Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. *Nat Biotechnol*. 2013; 31:142–147.
- Liu Z, Christensson M, Forslöv A, De Meester I, Sundqvist KG. A CD26-controlled cell surface cascade for regulation of T cell motility and chemokine signals. *J Immunol*. 2009;183:3616–3624.
- Lourenco, T. G. et al. Microbial signature profiles of periodontally healthy and diseased patients. *J Clin Periodontol*. 2014; 41:1027–1036.
- MacGregor AJ, Snieder H, Rigby AS, Koskenvuo M, Kaprio J, Aho K, Silman AJ. Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. *Arthritis Rheum*. 2000; 43, 30–37.
- Machtei EE, Hausmann E, Dunford R, Grossi S, Ho A, Davis G, Chandler J, Zambon J, Genco RJ. Longitudinal study of predictive factors for periodontal disease and tooth loss. *J Clin Periodontol*. 1999; 26: 374–380.
- Malmström V, Catrina AI, Klareskog L. The immunopathogenesis of seropositive rheumatoid arthritis: from triggering to targeting. *Nat Rev Immunol*. 2017; 17:60–75.
- Marcenes W, Kassebaum NJ, Bernabé E, Flaxman A, Naghavi M, Lopez A, Murray CJ. Global Burden of Oral Conditions in 1990-2010: A Systematic Analysis. *J Dent Res*. 2013; 592-597.
- Martins MD, Jiao Y, Larsson L, Almeida LO, Garaicoa-Pazmino C, Le JM, Squarize CH, Inohara N, Giannobile WV, Castilho RM. Epigenetic Modifications of Histones in Periodontal Disease. *J Dent Res*. 2015; 95: 215 – 222.
- McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nature Rev Immunol*. 2007; 7(6):429.
- Medzhitov R. Origin and physiological roles of inflammation. *Nature*. 2008; 454:428-436.
- Meng H, Ren X, Tian Y, Feng X, Xu L, Zhang L, Lu R, Shi D, Chen Z. Genetic study of families affected with aggressive periodontitis. *Periodontol* 2000. 2011;56:87-101.

- Merry K, Dodds R, Littlewood A, Gowen M. Expression of osteopontin mRNA by osteoclasts and osteoblasts in modelling adult human bone. *J Cell Sci.* 1993; 104:1013–1020.
- Mombelli A. Clinical parameters: biological validity and clinical utility. *Periodontol* 2000. 2005; 39: 30–39.
- Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol.* 2001; 19: 683–765.
- Mori G, Brunetti G, Colucci S, Oranger A, Ciccolella E, Sardone F, Pignataro P, Mori C, Karapanou V, Ballini A, Mastrangelo F. Osteoblast Apoptosis in Periodontal Disease: Role of TNF-Related Apoptosis- Inducing Ligand. *Int J Immunopathol Pharmacol.* 2009; 22:95-103.
- Murakami S, Mealey BL, Mariotti A, Chapple IL. Dental plaque–induced gingival conditions. *J Clin Periodontol.* 2018; 45:S17-27.
- Murray CJ, Vos T, Lozano R, Naghavi M, Flaxman AD, Michaud C, Ezzati M, Shibuya K, Salomon JA, Abdalla S, Aboyans V. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *The Lancet.* 2012; 380:2197-2223.
- Muto A, Mizoguchi T, Udagawa N, Ito S, Kawahara I, Abiko Y, Arai A, Harada S, Kobayashi Y, Nakamichi Y, Penninger JM. Lineage-committed osteoclast precursors circulate in blood and settle down into bone. *J Bone Miner Res.* 2011; 26: 2978–2990.
- Nagaraja C, Pradeep AR. Hepatocyte growth factor levels in gingival crevicular fluid in health, disease, and after treatment. *J Periodontol.* 2007; 78:742-747.
- Nelson RG, Shlossman M, Budding LM, Pettitt DJ, Saad MF, Genco RJ, Knowler WC. Periodontal disease and non-insulin dependent diabetes mellitus in Pima Indians. *Diabetes Care.* 1990; 13: 836–840.
- O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science.* 2010; 327:1098-1102.
- Okui T, Aoki Y, Ito H, Honda T, Yamazaki K. The presence of IL-17+/FOXP3+ double-positive cells in periodontitis. *J Dent Res.* 2010; 91:574-9.
- Okui T, Aoki-Nonaka Y, Nakajima T, Yamazaki K. The role of distinct T cell subsets in periodontitis—studies from humans and rodent models. *Current Oral Health Reports.* 2014; 1:114-23.
- Orima K, Yamazaki K, Aoyagi T, Hara K. Differential expression of costimulatory molecules in chronic inflammatory periodontal disease tissue. *Clin Exp Immunol.* 1999; 115:153-60.
- Orta-Mascaró M, Consuegra-Fernández M, Carreras E, Roncagalli R, Carreras-Sureda A, Alvarez P, Girard L, Simões I, Martínez-Florensa M, Aranda F, Merino R. CD6 modulates thymocyte selection and peripheral T cell homeostasis . *J Exp Med.* 2016; 213: 1387-1397.
- Ortega-Gómez A, Perretti M, Soehnlein O. Resolution of inflammation: an integrated view. *EMBO Mol Med.* 2013; 5:661-674.

- Padyukov L, Seielstad M, Ong RT, Ding B, Rönnelid J, Seddighzadeh M, Alfredsson L, Klareskog L, Epidemiological Investigation of Rheumatoid Arthritis (EIRA) Study Group. A genome-wide association study suggests contrasting associations in ACPA-positive versus ACPA-negative rheumatoid arthritis. *Ann Rheum Dis*. 2011;70, 259–265.
- Page RC, Schroeder H. Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest*. 1976; 34:235-49.
- Panezai J, Ghaffar A, Altamash M, Sundqvist KG, Engström PE, Larsson A. Correlation of serum cytokines, chemokines, growth factors and enzymes with periodontal disease parameters. *PloS One*. 2017; 12:e0188945.
- Panezai J, Ghaffar A, Altamash M, Engström PE, Larsson A. Periodontal disease influences osteoclastogenic bone markers in subjects with and without rheumatoid arthritis. *PloS One*. 2018; 13:e0197235.
- Patel RA, Wilson RF, Palmer RM. The effect of smoking on periodontal bone regeneration: a systematic review and meta-analysis. *J Periodontol*. 2012; 83: 143–155.
- Pelletier M, Maggi L, Micheletti A, Lazzeri E, Tamassia N, Costantini C, Cosmi L. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood*. 2010; 115:335-343.
- Petersen PE, Ogawa H. The global burden of periodontal disease: Towards integration with chronic disease prevention and control. *Periodontol 2000*. 2012; 60, 15–39.
- Peterson PE. Social inequalities in dental health. *Community Dent Oral Epidemiol*. 1990; 18: 153–158.
- Pianta A, Arvikar SL, Strle K, Drouin EE, Wang Q, Costello CE, Steere AC. Two rheumatoid arthritis-specific autoantigens correlate microbial immunity with autoimmune responses in joints. *J Clin Invest*. 2017;127:2946–2956.
- Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum*. 1995; 38:44-8.
- Rekedal LR, Massarotti E, Garg R, Bhatia R, Gleeson T, Lu B, Solomon DH. Changes in glycosylated hemoglobin after initiation of hydroxychloroquine or methotrexate treatment in diabetes patients with rheumatic diseases. *Arthritis Rheum*. 2010; 62:3569–3573.
- Rittling SR, Matsumoto HN, McKee MD, Nanci A, An XR, Novick KE, Kowalski AJ, Noda M, Denhardt DT. Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation in vitro. *J Bone Miner Res*. 1998; 13:1101–1111.
- Rogers A, Eastell R. Circulating osteoprotegerin and receptor activator for nuclear factor κ B ligand: clinical utility in metabolic bone disease assessment. *J Clin Endocrinol Metab*. 2005; 90: 6323-31.
- Rosenstein E, Greenwald R, Kushner L, Weissmann G. Hypothesis: the humoral immune response to oral bacteria provides a stimulus for the development of rheumatoid arthritis. *Inflammation*. 2004; 28: 311–318.

- Rubin E, Reisner HM, editors. Essentials of Rubin's pathology. Lippincott Williams & Wilkins; 2009.
- Scher JU, Szczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, Rostron T, Cerundolo V, Pamer EG, Abramson SB, Huttenhower C. Expansion of intestinal *Prevotella copri* correlates with enhanced susceptibility to arthritis. *eLife*. 2013; 2:e01202.
- Séguier S, Gogly B, Bodineau A, Godeau G, Brousse N. Is collagen breakdown during periodontitis linked to inflammatory cells and expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human gingival tissue? *J Periodontol*. 2001; 72 : 1398-1406.
- Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol*. 2008; 8(5):349.
- Shackleton B, Crawford F, Bachmeier C. Inhibition of ADAM10 promotes the clearance of A β across the BBB by reducing LRP1 ectodomain shedding. *Fluids Barriers CNS*. 2016; 13: 14-14.
- Shaju JP, Zade RM, Das M. Prevalence of periodontitis in the Indian population: A literature review. *J Indian Soc Periodontol*. 2011; 15:29-34.
- Shaw PJ, McDermott MF, Kanneganti TD. Inflammasomes and autoimmunity. *Trends Mol Med*. 2011; 17:57-64.
- Shiau HJ, Reynolds MA. Sex Differences in Destructive Periodontal Disease: A Systematic Review. *J Periodontol*. 2010; 81: 1379–1389.
- Simpson TC, Needleman I, Wild SH, Moles DR, Mills EJ. Treatment of periodontal disease for glycemic control in people with diabetes. *Aust Dent J*. 2010; 55:472-474.
- Smolen JS, Aletaha D, Barton A, Burmester GR, Emery P, Firestein GS ,Kavanaugh A , McInnes IB, Solomon DH, Strand V , Yamamoto K. *Nature Rev Dis Primers*. 2018; 4:18002:1-23.
- Smolen JS, Breedveld FC, Eberl G, Jones I, Leeming M, Wylie GL, Kirkpatrick J. Validity and reliability of the twenty-eight-joint count for the assessment of rheumatoid arthritis activity. *Arthritis Rheum*. 1995; 38: 38–43.
- Sodek J, Ganss B, McKee MD. Osteopontin. *Crit Rev Oral Biol Med*. 2000; 11:279–303.
- Sokolove J, Wagner CA, Lahey LJ, Sayles H, Duryee MJ, Reimold AM, Kerr G, Robinson WH, Cannon GW, Thiele GM, Mikuls TR. Increased inflammation and disease activity among current cigarette smokers with rheumatoid arthritis: a cross-sectional analysis of US veterans. *Rheumatology*. 2016; 55, 1969–1977.
- Solomon DH, Love TJ, Canning C, Schneeweiss S. Risk of diabetes among patients with rheumatoid arthritis, psoriatic arthritis and psoriasis. *Ann Rheum Dis*. 2010; 69:2114-2117.
- Stankov VS. Definition of inflammation, causes of inflammation and possible anti-inflammatory strategies. *Open Inflamm J*. 2012; 5: 1-9.
- Stathopoulou PG, Buduneli N, Kinane DF. Systemic biomarkers for periodontitis. *Curr Oral Health Rep*. 2015; 2:218-26.

- Strickland DK, Gonias SL, Argraves WS. Diverse roles for the LDL receptor family. *Trends Endocrinol Metab.* 2002; 13:66-74.
- Syversen SW, Gaarder PI, Goll GL, Ødegård S, Haavardsholm EA, Mowinckel P, Van Der Heijde D, Landewé R, Kvien TK. High anti-cyclic citrullinated peptide levels and an algorithm of four variables predict radiographic progression in patients with rheumatoid arthritis: results from a 10-year longitudinal study. *Ann Rheum Dis.* 2008; 67: 212–7.
- Taylor GW, Borgnakke WS, Graves DT. Association between Periodontal Diseases and Diabetes Mellitus. *Periodontal Disease and Overall Health: A Clinician's Guide.* Yardley, Pennsylvania, USA: Professional Audience Communications Inc. 2010.
- Taylor P, Gartemann J, Hsieh J, Creeden J. A systematic review of serum biomarkers anti-cyclic citrullinated peptide and rheumatoid factor as tests for rheumatoid arthritis. *Autoimmune Dis.* 2011; 815038-815038.
- Teeuw WJ, Kosho MX, Poland DC, Gerdes VE, Loos BG. Periodontitis as a possible early sign of diabetes mellitus. *BMJ Open Diabetes Res Care.* 2017; 5:e000326.
- Thorbert-Mros S, Larsson L, Berglundh T. Cellular components of long standing gingivitis and periodontitis lesions. *J Periodontol Res.* 2015; 50:535-543.
- Tobón GJ, Youinou P, Saraux A. The environment, geo-epidemiology, and autoimmune disease: rheumatoid arthritis. *J Autoimmun.* 2010; 1:10-14. .
- Ukai T, Yumoto H, Gibson FC, Genco CA. Macrophage-elicited osteoclastogenesis in response to bacterial stimulation requires Toll-like receptor 2-dependent tumor necrosis factor- α production. *Infect Immun.* 2008; 76: 812-9.
- Ursum J, Bos WH, van Dillen N, Dijkmans BA, van Schaardenburg D. Levels of anti-citrullinated protein antibodies and IgM rheumatoid factor are not associated with outcome in early arthritis patients: a cohort study. *Arthritis Res Ther.* 2010; 12:R8.
- Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4⁺ CD25^{hi} T-regulatory cells. *Blood.* 2006 ;108 :253-61.
- van den Broek T, Borghans JA, van Wijk F. The full spectrum of human naive T cells. *Nature Rev Immunol.* 2018:1.
- Van der Velden U, Abbas F, Armand S, Loos BG, Timmerman MF, Van der Weijden GA, Van Winkelhoff AJ, Winkel EG. Java project on periodontal diseases. The natural development of periodontitis: risk factors, risk predictors and risk determinants. *J Clin Periodontol.* 2006;33(8):540-8.
- Van Dyke TE, Dave S. Risk factors for periodontitis. *J Int Acad Periodontol.* 2005; 7:3-7.
- Viatte S, Massey J, Bowes J, Duffus K, arcOGEN Consortium, Eyre S, Barton A, Worthington J, Loughlin J, Arden N, Birrell F. Replication of associations of genetic loci outside the HLA region with susceptibility to anti-cyclic citrullinated peptide-negative rheumatoid arthritis. *Arthritis Rheumatol.* 2016; 68: 1603–1613.
- Welsing PM, Landewé RB, Van Riel PL, Boers M, Van Gestel AM, Van Der Linden S, Swinkels HL, Van Der Heijde DM. The relationship between disease activity and

- radiologic progression in patients with rheumatoid arthritis: a longitudinal analysis. *Arthritis Rheum.* 2004; 50:2082-2093.
- Weyand CM, Hicok KC, Conn DL, Goronzy JJ. The influence of HLA-DRB1 genes on disease severity in rheumatoid arthritis. *Ann Intern Med.* 1992; 117, 801–806.
- Whitney C, Ant J, Moncla B, Johnson B, Page RC, Engel D. Serum immunoglobulin G antibody to *Porphyromonas gingivalis* in rapidly progressive periodontitis titer, avidity and subclass distribution. *Infect Immun.* 1992; 60:2194-2200.
- Wilkinson RG. Health, hierarchy, and social anxiety. *Ann N Y Acad Sci.* 1999; 896:48-63.
- Younes R, Ghorra C, Khalife S, Igondjo-Tchen-Changotade S, Yousfi M, Willig C, Senni K, Godeau G, Naaman N. Pertinent cell population to characterize periodontal disease. *Tissue Cell.* 2009; 41:141-50.